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## SECTION MEETINGS

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|---|---|
| **CLEVELAND** | |
| Western Reserve University | March 13, 1942 |
| **ILLINOIS** | |
| University of Chicago | March 3, 1942 |
| **MINNESOTA** | |
| University of Minnesota | March 18, 1942 |
| **MISSOURI** | |
| Washington University | March 11, 1942 |
| **PACIFIC COAST** | |
| Mount Zion Hospital, San Francisco | March 4, 1942 |
| **SOUTHERN CALIFORNIA** | |
| Los Angeles Co. Medical Association | March 17, 1942 |

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### 13608

# Ultraviolet Light-Inactivated Antigens for Complement-Fixation Tests with Central Nervous System Virus Infections.

J. CASALS. (Introduced by L. T. Webster.)

*From the Laboratories of The Rockefeller Institute for Medical Research, New York City.*

The presence of complement-fixing antibodies has been described in the serum of individuals infected with any of a number of central nervous system viruses,[1] as well as a method for their determination that eliminates all non-specific reactions. A recent human epidemic of encephalitis was promptly diagnosed as due to Western equine encephalomyelitis virus by means of the complement-fixation reaction.[2]

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[1] Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

The antigens prepared for the complement-fixation test as previously described were virulent. In order to diminish the risk involved in the general use of such antigens, attempts were made to render them avirulent without materially destroying their antigenicity. This result has been accomplished by exposing the virus preparations to ultraviolet light for suitable length of time, as we shall describe.

The antigens were prepared in the following manner: Batches of 4-weeks-old W-Swiss mice were injected intracerebrally with one of the viruses; when prostrate these animals were sacrificed, their brains removed, weighed, and emulsified in 0.85% saline containing 2% normal guinea pig serum heated at 56°C for 20 minutes, the proportion being 1 g of infected brain to 10 cc of diluent. This emulsion was kept in the icebox for 20 hours and then centrifuged in a horizontal centrifuge at 2500 rpm for a half hour; the supernatant was frozen and thawed 5 times in a dry ice-alcohol mixture, and finally centrifuged in an angle head centrifuge[3] at 5000 rpm for 1 hour. The supernatant, following addition of merthiolate in a dilution of 1/10,000, constituted the antigen.

Antigens thus prepared with the viruses of rabies, Eastern and Western equine encephalomyelitis, lymphocytic choriomeningitis, and St. Louis encephalitis have been tested for virulence within 1 to 3 days of preparation by intracerebral injection into Swiss mice and found virulent in dilutions of $10^{-4}$ for Western equine encephalomyelitis, $10^{-3}$ for Eastern equine encephalomyelitis, $10^{-2}$ for rabies, lymphocytic choriomeningitis, and St. Louis encephalitis viruses respectively. Kept in the icebox the antigens retain their virulence for long periods of time.

Formalin- or heat-inactivation of antigens thus prepared was not satisfactory; formalin made them anticomplementary and heat destroyed their antigenicity. Ultraviolet light was used as a means of inactivation with very good results, as will be shown later. The source of ultraviolet light has been a mercury vapor lamp (Hanovia); the antigens to be inactivated were placed in quartz flasks before the addition of merthiolate and the flasks shaken mechanically while being irradiated. The time required to render each different antigen avirulent was determined by inoculating mice intracerebrally with the irradiated samples.

The avirulent irradiated antigens were then tested in parallel with

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[2] Casals, J., *Am. J. Public Health,* 1941, **31**, 1281.

[3] Pickels, E. G., *Review of Scientific Instruments,* in press.

TABLE I.

Effect of Irradiation with Ultraviolet Light on Complement-Fixing Antigens of Some Central Nervous System Viruses.

| Virus | Virulence of antigen before irradiation. Titer = 0.03 cc intracerebral dilution | Min. ultraviolet light irradiation to render antigens avirulent, min. | Anticomplementary power. Amt guinea pig serum 1:30, equivalent to 1 unit | | Specificity.* Serum titer | | Antigenicity. Antigen titer | |
|---|---|---|---|---|---|---|---|---|
| | | | With non-irradiated antigen, cc | With irradiated antigen, cc | With non-irradiated antigen. Homologous antigen§ | With irradiated antigen. Homologous antigen§ | Non-irradiated antigen | Irradiated antigen |
| E.E.E. | $10^{-3}$ | 90 | .16 | .20 | 1/128† | 1/128 | 1/2‡ | 1/2 |
| W.E.E. | $10^{-4}$ | 90 | .10 | .10 | 1/128 | 1/128 | 1/16 | 1/16 |
| LCM | $10^{-2}$ | 40 | .13 | .13 | 1/256> | 1/256> | 1/2 | 1/2 |
| St. Louis | $10^{-2}$ | 40 | .13 | .13 | 1/64 | 1/64 | 1/16 | 1/16 |
| Rabies (fixed) | $10^{-2}$ | 40 | .13 | .13 | 1/128 | 1/128 | 1/8 | 1/8 |

*Each serum was tested against its own antigen and the 4 heterologous antigens; the first dilution of serum was 1:2 or 1:4.

†Highest dilution of serum giving a 2+ or better fixation.

‡Highest dilution of antigen giving a 2+ or better reaction. Serum used in dilution of 1:24.

§Heterologous antigen all 0.

the non-irradiated ones with regard to their properties in the complement-fixation test, namely, anticomplementary power, specificity, and antigenicity; their stability, when kept in the icebox for several weeks, is being tested.

The results of these tests are shown in Table I and can be summarized as follows:

With our special set-up, periods of time of 90 minutes are required to destroy the virulence of the Eastern and Western equine encephalomyelitis antigens, and 40 minutes with rabies fixed, lymphocytic choriomeningitis, and St. Louis encephalitis antigens.

The irradiated antigens are not anticomplementary. The titer of the complement in the presence of irradiated and of non-irradiated antigen is the same.

As shown by the highest dilution of serum exhibiting a 2 plus or better reaction, the titer of a mouse hyperimmune serum against the irradiated and the non-irradiated antigen is the same. The specificity of the reaction with the irradiated antigen is as complete as with the non-irradiated one, as shown in both instances by the absence of any crossing with the heterologous antigens.

And finally, the titer of the antigen following irradiation is the same, or only slightly lower than the titer of the non-irradiated antigen; these titers were determined by testing serial twofold dilutions of antigen with a constant amount of immune serum.

In conclusion, by exposing virus suspensions of Eastern and Western equine encephalomyelitis, rabies, lymphocytic choriomeningitis, and St. Louis encephalitis to ultraviolet light, complement-fixing antigens can be obtained that are non-infective and specific, that lack an anticomplementary effect, and have a high antigenic titer.

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### 13609

## Effect of 2-4 Dinitrophenol on Rat Brain Respiration at 25, and 37.5°C.*

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*From the Department of Physiology, Stanford University.*

Measurements of the effects of graded concentrations of 2-4 dinitrophenol (DNP), at equilibrium, on oxygen consumption (concentration-action curves),[1] have been made on the intact rat[2] and on yeast.[3] However, satisfactory data of this sort do not appear to be available for mammalian tissue *in vitro*. In the present communication such data are furnished for cerebral cortex slices from adult male albino rats. Oxygen consumption was measured at 25° (±0.01°) and 37.5° (±0.01°) C. The methods used have been described previously,[4] therefore only certain features of the procedure, particularly relevant to the problem in hand, will be presented here. Conventional manometric methods (Warburg) were used.[5] The suspension medium was Ringer's-phosphate-glucose, pH 7.35, and all solutions added were of the same pH. The gas phase was oxygen. In each set of 14 manometers there was one control vessel receiving no DNP as well as a thermobarometer. DNP was added to the experimental vessels 30 minutes after the end of the thermoequilibration period. Thus in each case a 30-minute control period (used in the calculation of the stimulation ratio) preceded addition of the drug. Readings were taken every 10 minutes. The rate of oxygen consumption was constant again within 15 minutes after addition of DNP in concentrations evoking increased oxygen consumption and 30 minutes after addition of concentrations which inhibited oxygen consumption.

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* Supported by grants from the Markle Foundation and from the Fluid Research Fund of the Stanford University School of Medicine.

Respiration was measured in $\mu$l, N.P.T., per mg wet weight per hour ($Q_{O_2}$). In order to express the intensity of action of DNP on oxygen consumption, the stimulation ratio[6] was calculated for each concentration of DNP. This is the ratio of the metabolic rate under the influence of DNP ($Q_{O_2}$ DNP) to that which would exist if DNP were not present ($Q_{O_2}$ C). The values of $Q_{O_2}$ C used were taken from measurements of respiration preceding addition of DNP (control experiments showed that the respiration of rat brain cortex, under the conditions of these experiments, remains constant for several hours when no DNP is added). Thus the stimulation ratio may be written

$$\text{Stimulation ratio} = \frac{Q_{O_2}\ \text{DNP (after becoming constant)}}{Q_{O_2}\ \text{C}}$$

When this ratio is greater than unity, DNP is increasing oxygen

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[1] Clark, A. J., *Action of Drugs on Cells,* Baltimore, 1933; Clark, A. J., General Pharmacology, Vierter Band, *Heffter's Handbuch der Exp. Pharm.,* Berlin, 1937.

[2] Tainter, M. L., *J. Pharm. and Exp. Ther.,* 1934, **51**, 45.

[3] Field, J., 2d, Martin, A. W., and Field, S. M., *J. Cell. and Comp. Physiol.,* 1934, **4**, 405.

[4] Fuhrman, F. A., and Field, J., 2d, *J. Pharm. and Exp. Ther.,* in press.

[5] Dixon, M., *Manometric Methods,* London, 1934.

[6] Hall, V. E., Crismon, J. M., and Chamberlin, P. C., *J. Pharm. and Exp. Ther.,* 1937, **59**, 193.

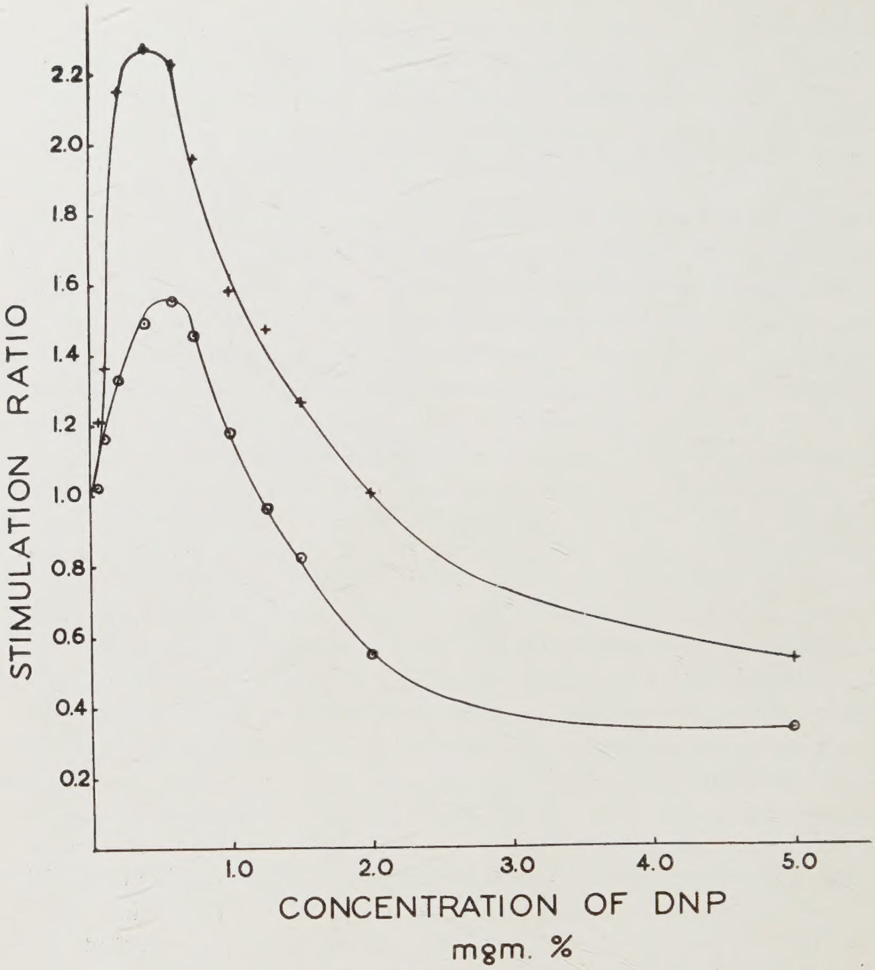

FIG. 1.

Graph showing the intensity of stimulation and inhibition (as the stimulation ratio) of oxygen consumption of excised rat cerebral cortex by graded concentrations of DNP. Crosses give values of the stimulation ratio at 25°C, circles at 37.5°C.

consumption, when less, oxygen consumption is diminished by the drug.

The results are given in Fig. 1, where the stimulation ratios at 25°C (crosses) and at 37.5°C (circles) are plotted as functions of DNP concentration (1.0 mg % sodium 2-4 dinitrophenoxide = $4.46 \times 10^{-5}$ M DNP). The fact that $Q_{O_2}$ DNP quickly became constant indicates that during this period of constancy equilibrium conditions obtained with respect to the distribution of DNP between the tissue slices and the suspension medium. The partition coefficient was not determined and for present purposes uniform distribution was assumed.

It is shown in Fig. 1 that the concentration of DNP corresponding to the maximum value of the stimulation ratio is nearly the same at 25°C and 37.5°C (0.4 mg % and 0.6 mg % respectively). However, the optimum concentration of DNP for stimulation of respiration in excised rat cerebral cortex at 37.5°C is much lower than the optimum concentration reported by Tainter[2] for stimulation of oxygen consumption in the intact rat (*ca* 6 mg %). This indicates either that the cerebral cortex is an atypical organ in this respect or that the experimental conditions were so different that direct comparison of the two findings cannot be made. Studies are in progress on other organs to clarify this point.

It is also shown in Fig. 1 that the maximum value of the stimulation ratio and the minimum inhibitory concentration of DNP are both considerably greater at 25°C than at 37.5°C. This is in concordance with our earlier observation[4] that the stimulation ratio corresponding to a single stimulating concentration of DNP (0.75 mg %) varies with temperature and increases with diminution in temperature over a wide range. It is also in accord with the findings of Hall, Crismon and Chamberlin[6] on the intact anesthetized cat.

*Conclusions.* The optimum concentration of DNP for the stimulation of oxygen consumption in excised rat cerebral cortex is approximately the same at 37.5°C and 25°C. However, the maximum value of the stimulation ratio and the minimum inhibitory concentration of DNP are considerably greater at 25° than at 37.5°C.

## 13610

## Oxygen Uptake of Blood Serum.*

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In connection with experiments in which solutions containing normal human blood serum were used, it was found that at pH values slightly below 7.0 the serum consumed $O_2$. This report is concerned with results obtained from experiments performed in an attempt to obtain further information concerning this phenomenon.

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* Aided by a grant from the Rockefeller Foundation for research in cellular physiology.

The serum was obtained from freshly drawn blood[†] in the usual manner and either used immediately or stored at 0°C for from 3 to 48 hours. The reaction was studied in Warburg manometers at 25°C. Buffers used were 0.5 M phosphate for pH values from 6.2 to 7.5 and Palitzsch's borax-boric acid mixtures for pH values from 7.5 to 8.5. One cc portions of serum from the same sample of blood showed almost identical values for $O_2$ consumption when buffered to the same pH (7.5) with either phosphate or borate. Each manometer cup contained either 1.0 cc or 2.0 cc of serum or dialysate, 0.2 cc of buffer, 0.1 cc of 10% KOH in the centerwell, and enough sterile 0.9% NaCl to make the total fluid volume 3.1 cc. Only serum or dialysate was omitted in the thermobarometer. When buffer was added from the side bulb of the flasks after temperature equilibration, measurable amounts of $CO_2$ were released, below pH 7.0, for at least 15 minutes after the pH was lowered. Therefore, buffer was added just before the flasks were attached to the manometers, and the first reading was taken 20 minutes later to allow time both for temperature equilibration and the evolution of $CO_2$. For this reason, the $O_2$ uptake measured below pH 7.0 was probably slightly smaller than the actual amount consumed.

The upper curve of Fig. 1 illustrates the time course of the $O_2$ uptake exhibited by 1.0 cc of serum at pH 6.5. The $O_2$ consumption is rapid during the first hour and is almost complete at the end of 90 minutes.

When serum is dialyzed in a cellophane tube against a large volume of 0.9% NaCl which is replaced several times during 24 hours, the serum then shows no $O_2$ uptake. However, if a given amount of serum is dialyzed against an equal volume of 0.9% NaCl, the dialysate and the serum each show an $O_2$ uptake which is just half that exhibited by an equal volume of undialyzed serum obtained from the same individual. The lower curve of Fig. 1 illustrates the rate of $O_2$ consumption of 1.0 cc of dialysate at pH 6.5. Dialysate heated for 15 minutes at 100°C (Table I) or boiled vigorously for 7 minutes shows the same $O_2$ uptake as the unheated control.

All samples of normal blood serum or dialysate showed an $O_2$ uptake when the pH was lowered; Table I summarizes the results obtained. The values at each pH range are averages of several determinations. At pH 6.2 to 6.6 the range of values for samples of serum from different individuals was from 30 to 70 $mm^3$ $O_2$ per cc of undialyzed serum and from 15 to 40 $mm^3$ $O_2$ per cc of dialysate. The

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[†] The blood used in all the experiments was made available by Dr. J. A. Greene of the University of Iowa Hospital.

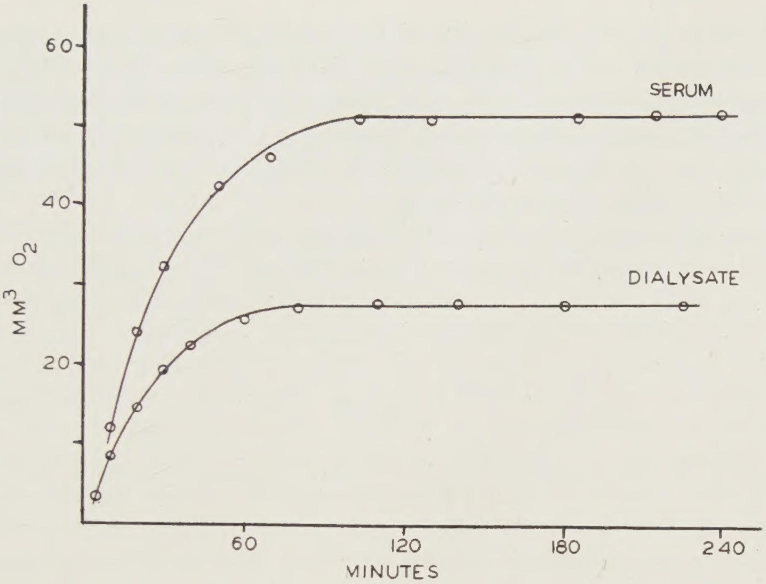

Fig. 1.

TABLE I.
Oxygen Uptake per cc of Serum or Dialysate.

| | pH 6.2-6.6 | | pH 7.0 | | pH 7.5-7.8 | | pH 8.1-8.5 | | Unbuffered pH 8.4 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. of Exp. | $Mm^3O_2$ | No. of Exp. | $Mm^3O_2$ | No. of Exp. | $Mm^3O_2$ | No. of Exp. | $Mm^3O_2$ | No. of Exp. | $Mm^3O_2$ |
| Serum | 19 | 49.7 | | | | | | | 3 | 1.3 |
| Dialysate | 18 | 23.3 | 2 | 13.4 | 4 | 2.8 | 4 | 0.9 | 2 | 0.0 |
| Heated | 6 | 15.2 | | | | | | | | |
| Control | 4 | 15.1 | | | | | | | | |
| Boiled | 1 | 21.3 | | | | | | | | |
| Control | 1 | 23.1 | | | | | | | | |

figures in the column at the extreme right were obtained from experiments in which either 1.0 cc or 3.0 cc samples of unbuffered serum or dialysate were used. The serum from 2 individuals with a moderate hypertension yielded values of 36.8 and 38.4 $mm^3$ $O_2$ per cc and one sample of serum from a hyperthyroid patient gave a value of 33.7 $mm^3$ $O_2$ per cc.

Ramsey and Warren[1] found that at physiological pH normal rabbit plasma consumed $O_2$ at a steady rate of 1.7 $mm^3$ per cc per hour and that a temporary increase or "burst" occurred when hemolyzed cells were added. They state that both the steady oxidation and the

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[1] Ramsey, Robert, and Warren, Charles O., Jr., *Quart. Exp. Physiol.*, 1934, **24**, 153.

"burst" are heat stable and only slightly cyanide sensitive, and suggest that the $O_2$ consumption is due to autoxidation of lipoids and proteins and may be catalyzed by the addition of pigments of the lysed cells. Canzanelli, *et al.*,[2] reported that horse serum consumed $O_2$ in small amounts at pH 10.5 and at a rate of about 50 $mm^3$ per cc per hour at pH 11.5 but none below that range.

Other workers have observed an $O_2$ uptake by plasma or serum but only under special conditions. Douglas[3] found that in the determination of the $O_2$ capacity of rabbit blood an absorption of $O_2$ occurred in alkaline plasma or serum when ferricyanide was added. More recently Litarczek[4] and Parsons and Parsons[5] have described a large $O_2$ uptake by alkaline rabbit plasma in the presence of ferricyanide but found no oxidation in its absence. They concluded that the $O_2$ uptake was due to oxidation of lipoids. Wright and Arthur[6] have made a further study of the reactions involved in such alkaline plasmas and their results indicate that unsaturated fatty acids may be largely responsible for the oxidation in the presence of ferricyanide. They showed, further, that, after dialysis of serum in a collodion sac, only about one-third of the original $O_2$ uptake was accounted for by dialyzable substances. Harris[7] showed that plasma irradiated with ultraviolet light consumed $O_2$ and Smetana[8] described a photo-oxidation of serum and serum proteins in the presence of hematoporphyrin as a sensitizer.

The small total $O_2$ consumption observed in slightly alkaline serum dialysate (Table I) seems somewhat comparable to the small rate of oxidation which Ramsey and Warren[1] found in rabbit plasma. They state, however, that this oxidation continued for several hours at nearly the same rate. The $O_2$ consumption of human serum and serum dialysate in acid solution and in the absence of oxidizing agents is of considerably smaller magnitude than that observed by Canzanelli, *et al.*,[2] for strongly alkaline horse serum and by several workers for alkaline rabbit plasma containing ferricyanide. Whether these facts mean (a) that the materials being oxidized under the various conditions are different, or (b) that the addition of an oxidizing agent enhances, in slightly alkaline solution, an oxidation that may take place spontaneously in acid or very strongly alkaline solution, the present series of experiments does not indicate. If fatty acids undergo oxidation in human serum at acid reaction, as it seems likely that they do in alkaline rabbit serum in the presence of ferricyanide (Wright and Arthur[6]), they must be dialyzable.

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[2] Canzanelli, Attilio, Greenblatt, Milton, Rogers, Gertrude A., and Rapport, David, *Am. J. Physiol.*, 1939, **127**, 290.

[3] Douglas, C. G., *J. Physiol.*, 1910, **39**, 453.

[4] Litarczek, G., *J. Physiol.*, 1928, **65**, 1.

[5] Parsons, T. R., and Parsons, W., *Biochem. J.*, 1927, **21**, 1194.

[6] Wright, G. P., and Arthur, B., *J. Biol. Chem.*, 1931, **90**, 757.

[7] Harris, D. T., *Biochem. J.*, 1926, **20**, 271.

[8] Smetana, Hans, *J. Biol. Chem.*, 1938, **124**, 667.

*Summary.* An $O_2$ uptake is observed when the pH of normal human blood serum or its dialysate is lowered. Heated dialysate shows the same $O_2$ uptake as unheated dialysate. All of the material oxidized at acid reaction is dialyzable.

### 13611

## Survival Time of Pregnant and Nonpregnant Rats after Bilateral Nephrectomy.*

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With total anuria, the duration of life is dependent upon the rate of development of a toxic factor which, in animals otherwise normal, has been shown by Hoff, Smith and Winkler[1] to be potassium. The cause of death after bilateral nephrectomy is not the same as the cause of death in uremia of nephritic origin, for in the latter state, in the absence of total anuria, the terminal potassium ion concentration does not reach fatal levels.

Bergman and Drury,[2] studying the survival time of normal fasting rats after bilateral nephrectomy state: "The low variability in the survival time of the standard nephrectomized rat under any well defined set of conditions makes it easy to determine whether any given factor or procedure is harmful or beneficial in renal insufficiency." They show that the survival time is shortened by such extraneous procedures as the administration of water or protein. In such experiments death is due to the accumulation of potassium, and the toxicity of meat was shown to be due to its high potassium content.[1, 2, 3]

Any factor which increases the rate of accumulation of potassium or causes the production of some other more toxic product which

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* This work was aided by the John and Mary Markle Foundation.

[1] Hoff, Hebbel E., Smith, Paul K., and Winkler, Alexander W., *J. Clin. Invest.*, 1941, **20**, 607.

[2] Bergman, Hyman C., and Drury, D. R., *J. Clin. Invest.*, 1939, **18**, 777.

[3] Addis, T., and Lew, W., *J. Clin. Invest.*, 1939, **18**, 773.

would normally be excreted into the urine might be expected to shorten the life of nephrectomized animals. The present work was undertaken to investigate the possibility that pregnancy constitutes such a factor. A shortening of survival time would offer support and elucidation to the commonly expressed view that impaired kidneys cannot carry the extra "load" imposed by pregnancy.

*Procedure.* Twenty-nine normal nonpregnant female rats weighing between 130 and 181 g were subjected to a fast of 24 hours, as recommended by Bergman and Drury, and both kidneys were removed through small lumbar incisions under ether anesthesia. No further food or water was given. After nephrectomy, each animal was placed in a suspended cage connected with a lever which recorded any movements on a continuous kymographic record. During the

TABLE I.
Survival Time of Nephrectomized Rats.

| Group I | | | Group II | | |
|---|---|---|---|---|---|
| Stage of pregnancy, days | Pregnant N-19 Survival time, hr | Nonpregnant N-19 Survival time, hr | Stage of pregnancy, days | Pregnant N-20 Survival time, hr | Nonpregnant N-10 Survival time, hr |
| 14 | 34 | 86 | 14 | 50.50 | 78.50 |
| " | 51.5 | 49 | " | 50.00 | 51.75 |
| " | 71.5 | 40 | " | 62.50 | 74.00 |
| " | 72 | 36 | " | 84.00 | 99.00 |
| " | 42.5 | 60 | " | 81.25 | 80.00 |
| " | 72 | 70 | " | 108.00 | 71.50 |
| " | 30 | 70 | " | 67.00 | 61.00 |
| " | 54 | 76 | 15 | 60.00 | 67.75 |
| " | 48 | 72 | " | 57.25 | 103.25 |
| " | 36 | 75.5 | " | 69.00 | 73.50 |
| " | 54 | 60 | " | 70.50 | |
| " | 41.5 | 45 | " | 80.00 | |
| " | 55 | 62 | " | 66.00 | |
| " | 87 | 77 | 17 | 62.00 | |
| " | 64 | 55 | 18 | 72.00 | |
| " | 33 | 69 | " | 65.50 | |
| " | 72 | 58 | " | 72.00 | |
| " | 72 | 54 | 19 | 67.00 | |
| " | 76 | 78 | " | 69.00 | |
| | | | " | 57.00 | |
| Median | 55.00 | 62.00 | | 67.00 | 73.75 |
| Mean | 56.10 | 62.76 | | 68.50 | 76.00 |
| $\sigma$ dist. | 16.25 | 13.86 | | 12.71 | 16.37 |
| S.E. mean | 3.72 | 3.17 | | 2.84 | 5.18 |
| Mn. diff. | 6.66 | | | 7.5 | |
| $\sigma$ diff. | 5.03 | | | 6.2 | |
| Critical ratio: $\frac{\text{Mn. diff.}}{\sigma\text{ diff.}}$ | 1.32 | | | 1.2 | |

night, or while not under direct observation, the rat received a mild faradic stimulus at intervals of 15 minutes; so that if it was alive the resultant body movements were recorded. In this way the time of death could be ascertained to within 15 minutes.

Thirty-nine rats of the same age and weight were subjected to the identical procedure during pregnancy. Of these, 32 were at the 14th or 15th day of pregnancy when nephrectomized and 7 at the 17th, 18th and 19th days.

*Observations.* The results of the experiment are presented in Table I. It will be noted that the data are subdivided into two groups. After the completion of the work on Group I minor modifications in technic were introduced to facilitate the collection of further data. Group II experiments were then performed. The analysis presented shows that although this technical change prolonged the survival time, it did not produce a significant difference between the observations on pregnant and nonpregnant animals.

The average survival times for the nonpregnant control rats were: Group I, 62.76 ± 3.17; Group II, 76.00 ± 5.18 hours, and for the pregnant rats: Group I, 56.1 ± 3.72; Group II, 68.50 ± 2.84 hours. The median values are shown on the table. The standard errors of the differences between the two means were 5.03 and 6.20 respectively. Since the observed differences of 6.66 and 7.50 hours between the means are scarcely greater than the standard errors of the differences, these differences may well be chance occurrences quite independent of pregnancy. It will be noted by way of comparison that the administration of water[2] results in a shortening of survival time from 81 to 67 hours.

Differences between the survival times of the animals at different stages of pregnancy were not evident in this experiment. None of the animals here reported went into labor.

There were no observed differences in the clinical behavior of the pregnant and nonpregnant rats after bilateral nephrectomy. Autopsies led us to believe that the fetuses survived as long as the mother, although it was noted that fetal body growth had apparently ceased soon after the maternal kidneys were removed.

*Conclusions.* In this series we find no statistically significant difference between survival time of pregnant and nonpregnant rats after bilateral nephrectomy in these groups of animals. These observations, therefore, lend no support to the concept that pregnancy is a burden on the kidneys. Even if the observed differences of survival time of 7 hours are in fact not due to chance, their magnitude is very small.

### 13612

## Relation of Fasting Ketosis to Nature of Protein and Fat Content of Preceding Diet.

Eaton M. MacKay, Frank E. Visscher and Arne N. Wick.

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It has been shown that the rapidity of onset and the degree of ketosis reached during fasting bears an inverse relation to the protein content of the preceding diet,[1, 2] apparently because this determines the amount of "stored" protein available for catabolism and thus for the formation of antiketogenic material (glucose) during fasting.[2] The present study was designed to examine the influence of the nature of the protein and of the fat content of the preceding diet upon a fasting ketosis.

The experimental and analytical methods used were similar to those which have been described before.[2] The degree of ketosis was measured by the height of the blood ketone body concentration as well as by the urine excretion of ketone bodies in one experiment. Details of the experiments are presented in Tables I and II.

*Nature of Protein Intake.* The onset was more rapid and the degree of ketosis reached definitely higher when the protein of the preceding diet was the incomplete and biologically poor protein, gelatin (Exp. 1, Table I). Blood albumen feeding led to the lowest fasting ketosis of the four proteins studied. Edestin and casein gave similar results, the degree of ketosis being less than that found after gelatin feeding and greater than that following the blood albumen diet. The urine nitrogen figures indicate that the degree of ketosis is influenced by feeding these proteins through the amount of body protein they make available for catabolism during the fasting period. In this the effect is the same as the result of feeding varying amounts of the same protein.[2] The amount of antiketogenic material—glucose from the protein being catabolized—appears to be the all-important factor determining the extent of a fasting ketosis.

*Nature of the Fat Intake.* We failed to find any significant influence upon a fasting ketosis of the amount of fat in the preceding diet. The influence of the nature of the preceding fat intake has been studied by feeding a number of natural fats. The feeding of

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[1] MacKay, E. M., Carne, H. O., and Wick, A. N., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 40.

[2] MacKay, E. M., Carne, H. O., Wick, A. N., and Visscher, F. E., *J. Biol. Chem.*, 1941, **141**, 889.

TABLE I.
*Experiment 1—Influence of Nature of Protein in †Preceding Diet upon Fasting Ketosis.

| Diet group | Hrs of fasting | Liver glycogen, % | Blood ketones, mg% | Urine excretion per sq. dm. body surface per day | |
|---|---|---|---|---|---|
| | | | | mg acetone | mg N |
| 1 Gelatin | 0 | 1.60 | 3 | | |
| | 12 | .06 | 20 | tr. | 20 |
| | 24 | .05 | 41 | 1 | 20 |
| | 48 | .04 | 54 | 18 | 19 |
| | 72 | .02 | 53 | 32 | 17 |
| 2 Edestin | 0 | .30 | 3 | | |
| | 12 | .04 | 15 | tr. | 26 |
| | 24 | .03 | 22 | 1 | 24 |
| | 48 | .05 | 30 | 5 | 22 |
| | 72 | .02 | 41 | 17 | 21 |
| 3 Casein | 0 | .82 | 5 | | |
| | 12 | .08 | 12 | tr. | 26 |
| | 24 | .05 | 24 | 1 | 25 |
| | 48 | .04 | 34 | 14 | 24 |
| | 72 | .03 | 44 | 17 | 22 |
| 4 Albumen | 0 | .40 | 4 | | |
| | 12 | .03 | 17 | tr. | 23 |
| | 24 | .04 | 26 | tr. | 25 |
| | 48 | .06 | 29 | tr. | 25 |
| | 72 | .05 | 33 | 6 | 26 |

*Fifteen female albino rats 120 days old were placed on the special diets for 10 days; 3 were sacrificed for liver glycogen and blood ketone determinations at each point where observations are recorded. The rats averaged 150 g in body weight when placed on the diets and 147 (grp. 1), 160 (grp. 2), 165 (grp. 3) and 155 (grp. 4) g respectively at the end of 10 days. Food was removed from the cages 14 hours before the recorded fasting periods were begun.

†The diets contained sucrose 50, salt mixture 5, brewer's yeast 5, cod-liver oil 5 and hydrogenated vegetable oil 20 (see ref. [3] for details of these ingredients), plus 15 parts of the protein ingredient. The gelatin was a high grade finely powdered food product, the edestin Pfanstiehl's highest purity, the casein "Labco" brand vitamin free casein, and the albumen an Eimer & Amend product termed Albumen from blood, C.p. The food intakes on the 4 diets were 6.7, 7.7, 8.4 and 6.2 g per rat per day respectively.

none of these appeared to influence directly the subsequent fasting ketosis. After feeding 7 of the fats (Table II, diet grps. 1-7), namely cottonseed oil, cocoanut oil, corn oil, cod-liver oil, beef fat, butter fat, and mutton fat, there was a high and rapidly developing fasting ketosis. This was accompanied by fairly low rates of nitrogen excretion, indicating that no large amount of antiketogenic material was being formed from catabolized protein. The degree of fasting ketosis was much less when pork fat, cocoa butter, and olive oil (Table II, grps. 8, 9 and 10) had been fed. This lesser ketosis was accompanied by higher rates of nitrogen excretion. It would seem obvious that here the nature of the fat in the preceding diet

TABLE II.
*Experiment 2—Influence of Nature of Fat in Preceding Diet upon Fasting Ketosis.

| Group diet | Food intake, g per sq. dm. body surface per day | Body weight | | Blood ketone bodies during fasting mg% | | | | Urine N excretion during fasting in mg per sq. dm. body surface per day | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Start of fasting g | End of fasting g | 0 hrs | 24 hrs | 48 hrs | 72 hrs | 24 hrs | 48 hrs | 72 hrs |
| 1. Cottonseed Oil† | 1.82 | 247 | 230 | 1.3 | 20 | 35 | 44 | 19 | 21 | 20 |
| 2. Cocoanut Oil | 1.68 | 243 | 230 | 2.8 | 20 | 33 | 44 | 16 | 23 | 20 |
| 3. Corn Oil‡ | 1.71 | 277 | 262 | 2.3 | 19 | 37 | 39 | 16 | 22 | 20 |
| 4. Cod-liver Oil | 2.06 | 263 | 250 | 1.4 | 19 | 40 | 36 | 18 | 24 | 20 |
| 5. Beef Fat | 1.60 | 243 | 234 | 1.4 | 16 | 24 | 40 | 19 | 21 | 23 |
| 6. Butter Fat | 1.53 | 269 | 256 | 1.3 | 17 | 39 | 35 | 16 | 23 | 21 |
| 7. Mutton Fat | 1.70 | 238 | 228 | 1.0 | 16 | 30 | 34 | 15 | 26 | 22 |
| 8. Pork Fat | 1.81 | 287 | 271 | 1.2 | 13 | 20 | 25 | 23 | 28 | 30 |
| 9. Cocoa Butter | 1.79 | 281 | 269 | 1.0 | 19 | 26 | 22 | 16 | 25 | 27 |
| 10. Olive Oil | 2.04 | 260 | 249 | 1.2 | 12 | 10 | 17 | 19 | 26 | 30 |
| 11. Hydrogenated Vegetable Oil§ | 2.06 | 260 | 250 | 1.4 | 18 | 34 | 25 | 19 | 21 | 18 |

*Adult male rats were placed upon the special diets for 20 days after a 6-day period of fasting. The food intake was recorded during the 10-day period preceding fasting and calculated upon the body weight (from which the body surface was computed) on the last day of the feeding period. The diets fed were composed of sucrose 40, casein 5, brewer's yeast 5, Standard salt mixture 5, powdered cellulose ("Cellu Flour") 5, and 40 parts of the respective fats.

†Wesson Oil.

‡Mazola.

§Crisco.

affects the subsequent fasting ketosis only as it may influence the amount of body protein which is catabolized and thus gives rise to antiketogenic material (glucose) during fasting. Our data do not indicate how a difference in the nature of the fat in the diet might influence protein storage and its subsequent rate of catabolism.

The results with a processed fat—hydrogenated vegetable oil (Crisco, Table II, grp. 11)—were interesting. The degree of fasting ketosis was lower than would be expected with such a low level of nitrogen excretion. It would be desirable to compare the ketosis after feeding an untreated and a hydrogenated vegetable oil because of the known peculiar characteristics of the latter.

*Summary.* The degree of fasting ketosis was influenced by the nature of the preceding protein intake. The ketosis bore an inverse relation to the protein catabolism during fasting, being higher after gelatin than after blood albumen feeding. Casein and edestin produced an intermediate fasting level of nitrogen excretion and ketosis.

Various natural fats fed prior to fasting were without influence upon the degree of fasting ketosis except as the protein catabolism varied. The usual inverse relation between the ketosis levels and the amount of nitrogen excreted was evident.

### 13613

# Comparison of Efficiency-Increasing and Toxic Effects of Theophyllinated Scillaridin* on Isolated Cat Heart.†

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Recently a new material prepared by replacing the sugar moiety in squills glycoside with 2 molecules of theophylline per molecule of genin has been made available for study.

Two reports[1, 2] dealing with its toxicity indicate that the material is appreciably less toxic than either the genin alone, or the glycoside, though the toxic effects, qualitatively, are essentially similar.

The general plan and logic of the experimental procedure has already been discussed.[3] Although the investigation cannot be considered complete, it is deemed worthwhile to make the present data available now because the new drug compares so favorably with the accepted cardiotonics, as judged by similar criteria, and because further studies on this problem have been indefinitely postponed.

Twenty-one experiments were done with the completely isolated cat heart, according to a method previously described.[4] Diastolic volume was recorded continuously in all experiments, and coronary flow in 5. The drug was administered dissolved in water, 1 mg per cc.

A dose causing a decrease in diastolic volume was accepted as exerting an efficiency effect. The onset of persistent irregularities within 4 hours of the time of administration of the drug constituted the criterion for toxicity. Experiments in which cardiac failure before the 4-hour period placed the interpretation in doubt were discarded.

The results are presented in Table I. In experiment 29, a dose of 1.5 mg/100 g produced a prolonged efficiency effect, irregularities appearing just at the close of the 4-hour period. In experiment 18 the same dose produced a marked efficiency effect uncomplicated by

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* Furnished by Parker Dorn, Inc., Worcester, Mass.

† Assistance in the preparation of these materials was furnished by the personnel of the Work Projects Administration, Official Project No. 165-1-71-124, Sub-project No. 392.

[1] Riseman, J. E., and London, S. B., *Science*, 1940, **92**, 384.

[2] DeGraff, A. C., and Lehman, R. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 36.

[3] Moe, G. K., and Visscher, M. B., *J. Pharm. and Exp. Therap.*, 1938, **64**, 65.

[4] Lorber, V., *Am. Heart J.*, in press.

TABLE I.

| Exp. No. | Dose in mg/100 g of ventricle | *Duration of vol. effect, min. | Onset of irregularities, min. | Remarks |
|---|---|---|---|---|
| 5 | 36.0 | 30 | 20 | Efficiency effect with toxic manifestations |
| 10 | 12.4 | 127 | 127 | ” |
| 11 | 5.2 | 128 | 217 | ” |
| 29 | 1.5 | 225 | 239 | Minimum irregularity dose (C.I.D.) |
| 18 | 1.5 | 240 | Regular rhythm at 4 hours | Efficiency effect without toxic manifestations |
| 20 | 0.96 | 240 | ” | ” |
| 26 | 0.65 | 210 | ” | ” |
| 13 | 0.41 | 180 | Failed before 4 hours | Minimum efficiency dose (C.E.D.) |
| 17 | 0.38 | Questionable vol. effect | Regular rhythm at 4 hrs | Probably no effect |
| 14 | 0.1 | None | Discontinued at 3½ hrs. Regular rhythm | No effect |

*Measured to the point of least diastolic volume.

toxic manifestations. Accordingly this dose is taken as approximating the minimum cardiac irregularity dose (C.I.D.) or least dose that will produce toxic irregularities within 4 hours.

Below this level the efficiency effect was the only one noted. The minimum cardiac efficiency dose (C.E.D.), or least dose producing an efficiency effect was found to be 0.41 mg/100 g. Though failure occurred before 4 hours in this experiment, it could be accepted since the dose was considerably smaller than the C.I.D. It should be stated that in the experiments discarded because of early failure, the relationship between dose and efficiency effect was not as regular as noted in the other experiments, some relatively large doses causing trivial volume changes. This can probably be ascribed to the poor condition of the heart in these experiments. In the main, however, the deleted experiments support the tabulated results.

The relationship of the efficiency-increasing and toxic effects of the drug on the isolated heart can be expressed in the ratio C.I.D/C.E.D., a measure of the margin of safety of the drug. The numerical value of the ratio in these experiments is 3.65, compared with values of 2.46, 1.04, and 2.00, for ouabain, K strophanthosid, and lanatosid C, respectively, determined on the isolated cat heart in similar experiments.[5] This suggests a wider margin of safety for the theophyllinated scillaridin, as judged by these criteria.

The expectation that inclusion of the theophylline molecule in the new preparation might lead to enhanced coronary flow has not been fulfilled under the conditions of this study. In the 5 experiments in which coronary flow was measured, there was no effect in 3 cases, a slight decrease in flow in one, and a slight increase in one. The dose in the last case was below the C.E.D.

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[5] Moe, G. K., unpublished data.

*Summary.* A new cardiac drug, a dimethylxanthine genate, has been studied in the completely isolated cat heart. It has been found to exhibit marked cardiotonic properties, and low toxicity. It exerts no appreciable coronary dilator effect on the completely isolated heart.

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### 13614

## Antibody Response of Persons with Pellagra, Beriberi and Riboflavin Deficiency.*

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During the past two decades, extensive animal experimentation has shown that in certain instances a direct relationship exists between the severity of an induced infection and the nutritional state of the animal. In a large group of persons with naturally occurring pellagra, beriberi, and riboflavin deficiency, Riddle, Spies and Hudson[1] found a depression of the blood's bactericidal power for streptococci and staphylococci, and demonstrated a characteristic flora of hemolytic strains of these organisms from oral and ocular lesions in riboflavin deficiency. The present report is concerned with observations on the relationship between these deficiency states in human beings and the response of their immune systems to antigenic stimulation.

*Materials and Methods.* Fifty patients were selected from the Nutrition Clinic of the Hillman Hospital, Birmingham, Alabama, using the following criteria:

1. A history indicating that their economic status and food-habits made it unlikely that they would or could change their diet significantly during the 5-month period of observation from May to Sep-

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* This study was aided by a grant from the National Foundation for Infantile Paralysis, Inc., and by a grant from Mead Johnson and Company of Evansville, Indiana.

[1] Riddle, Jackson W., Spies, Tom D., and Hudson, N. Paul, PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 361.

tember. 2. Some evidence of nutritional failure. The patients were classified as to the type of disease and the degree of severity. Group I included 17 patients, 7 of whom had symptoms of mild deficiency disease, and 10 undernourished patients who presented no clinical signs of a deficiency at the time of the study, but who had previously exhibited these symptoms. Group II included 23 patients who had diagnostic lesions of moderately severe deficiency disease. Group III included 8 patients with clinical evidence of rather severe pellagra.

3. An absence of *Bacterium tularense* agglutinins as evidenced by negative reactions of the serum prior to injection of antigen. Suspensions of an avirulent strain of the tularense organism—No. 38 Francis—grown on gluco-cystine blood-agar and washed with physiological saline were prepared at weekly intervals; 0.5 cc was added to 1 cc of serum-dilutions ranging from 1:10 to 1:640.

4. An absence of *Brucella melitensis* agglutinins shown by negative reactions of the serum. (This was to eliminate the possibility of cross-reaction with the tularense antigen.)

Each patient was injected intramuscularly with a formalin-killed detoxified suspension of several strains of *B. tularense,* which was prepared and standardized by Mr. William Hesselbrock in the laboratory of Dr. Lee Foshay, University of Cincinnati. The injections were given on 3 successive days to avoid the reactions that injections of the tularense organism sometimes produce when given at longer intervals. On completion of the injections, agglutinative tests of the sera were done at weekly intervals. The brief interval between injections insured responses of a primary nature allowing for observation of the fall in titer over a relatively short period. At monthly intervals agglutinin-nitrogen determinations were made according to the method of Heidelberger and Kabat.[2] The bacteria for these determinations were grown on gluco-cystine blood-agar, washed with physiological saline and preserved with 0.01% merthiolate. The suspension contained 0.525 mg nitrogen per cc. One cc was added to 0.5 cc of serum and the mixture was incubated at 37°C for 2 hours. After overnight chilling, the sediment was packed and washed and the Kjeldahl determinations were made.

Our antigens and technics were identical with those used by Foshay and Hesselbrock in a series of persons with no clinical evidence of deficiency disease. These investigators have kindly allowed the use of their unpublished results to compare with our findings in persons with nutritional deficiencies.

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[2] Heidelberger, Michael, and Kabat, Elvin A., *J. Exp. Med.,* 1934, **60**, 643.

One of our associates who had received the Foshay suspension two months earlier furnished serum for a positive control.

*Observations.* In the control group, the agglutinative titer after injection ranged from 1:20 to 1:1280 with a large majority between 1:80 and 1:160. The length of time required for this response to appear varied from 12 to 40 days after the initial stimulation. While the period of titer-maintenance varied, agglutination could be detected in various dilutions a year after vaccination in 50% of these persons. The positive control serum was constant at 1:80 throughout the period of study.

In our patients with deficiency-disease, the time required for a response to appear varied from 9 to 30 days following the first injection of the suspension. The period over which the titer was maintained deserves special note, since in all cases it was significantly less than in normal persons. In no instance was there a positive reaction after 64 days and in some of the severe cases the agglutinins disappeared in as short a time as 7 to 10 days from the time of the highest response. In the more severe cases the maintenance-time was lessened in accordance with the severity of the deficiency.

The maximal titer obtained varied in the 3 groups. In Group I they ranged from 1:40 to 1:320/640 and were well within the normal range reported by Foshay.[3] In Group II the general response was from 1:20 to 1:40, and fell somewhat below the normal variation. In Group III 2 of the patients had titers of 1:20, while the remaining cases in this group had a peak of only 1:10.

The series of agglutinin-nitrogen determinations suggested a direct proportion between the nitrogen and the titers above 1:10. Fall in titer was accompanied by a corresponding decrease in detectable antibody-nitrogen.

*Summary.* Response to antigenic stimulation with *B. tularense* in persons with pellagra, beriberi, and riboflavin-deficiency was less than in normal persons. In general, the resulting agglutinative titers were less in proportion to the severity of the deficiency, and persons with the greatest deficiency had less ability to maintain their titers. There was a quantitative relation between the higher titers and the amount of agglutinin-nitrogen.

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[3] Foshay, Lee, unpublished observations.

### 13615

# Spontaneous Ornithosis (Psittacosis) in Chickens the Cause of a Human Infection.

K. F. Meyer and B. Eddie.

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On December 5, 1940, Dr. Albert W. Van Sickle, Morristown Memorial Hospital, Morristown, New Jersey, kindly submitted for examination the blood serum of a 54-year-old wife of a poultry farmer, who had during the past 2 years lost about 500 chickens from some unknown disease. On November 23 she complained of severe headache, joint pains and grippy-like feelings; her temperature was 102°F, pulse 70 and respirations 20. She had severe chills at 4- to 5-hour intervals. On admission to the hospital with a temperature of 105°F, the X-ray examination on the 28th revealed an early pneumonitic consolidation within the right upper lobe without cough or sputum and a W.B.C. of 7,000. Throat cultures produced no pneumococci and despite sulfathiazole treatment the temperature remained *a continua* at 102 to 103°F. The serum specimens gave a strongly positive reaction in the complement fixation test for psittacosis in a dilution of 1:32 on the 12th, >1:128 on the 41st and 155th days. A few flakes of mucus secured from the buccal cavity on the 32nd day of her illness and after her return from the hospital failed to infect mice. Convalescence was uneventful but slow.

An epidemiological investigation kindly undertaken by the Department of Health of the State of New Jersey conclusively excluded an exposure of the patient to psittacine or any other domesticated birds except chickens. Through the courtesy of Mr. H. R. Nicolas of the Department on January 6th, 1941, 3 chickens were obtained and sent in a frozen state to California. The autopsies revealed no gross lesions suggestive of psittacosis. Although the cadavers showed definite emaciation and anemia, organ emulsions of the spleen, liver and kidneys in pools and separately were inoculated intraperitoneally into mice. The pool from 1 chicken (7754; splenic measurements 15x10 mm) produced in the sacrificed mice by the 20th day definitely enlarged spleens with inconclusive microscopic findings. On the third intraperitoneal passage, aside from splenic enlargement, the blood tinged purulent peritoneal exudate contained numerous polyblasts with few but typical *Microbacterium multiforme* ornithosis (MMO) readily demonstrated in smears stained by the Castaneda and the

Macchiavello methods. A mouse of the fourth passage, sacrificed on the 10th day, showed a much larger number of MMO bodies; in fact, a culture was obtained in the Li-Rivers and Zinsser-Fitzpatrick-Wei media. On intracranial and intranasal injections of 0.03 cc of the exudate, either paralysis with death due to a choriomeningitis on the 8th day or a lobar pneumonia with death on the 11th-12th day was regularly induced in mice and in hamsters. The smears from the lesions were invariably teeming with elementary bodies. Neither the intraperitoneal nor subcutaneous injections of the virus in concentration of one billion infective units estimated microscopically are capable of causing with any regularity fatal infections in a strain of mice highly susceptible for the psittacine viruses. In the non-fatal infections the agent has persisted in the liver and spleen for months. The comparative pathogenicity tests of the chicken virus for birds inoculated by various routes are summarized in Table I.

The chicken virus is indistinguishable from the more recently isolated pigeon viruses; it produces on intramuscular injection in chickens a chronic wasting disease with latency in the spleen, liver and kidneys. Ricebirds and parrakeets are readily infected; intracranial infections in avian species produce fatal meningitides. Antigenically the Microbacterium appears to be identical with two parrakeet viruses with which it has been compared. In the complement fixation test the agent is indistinguishable from the psittacine viruses.

During the latter part of April and during the month of May with the courteous assistance of Mr. W. H. MacDonald of the New Jersey Health Department, which is herewith gratefully acknowledged, the poultry farm was repeatedly visited and the epidemiologic inquiries extended. For the past 5 years the family consisting of husband and wife and 3 children have raised White Leghorn pullets. They claim that in 1939-1940 approximately 500 chickens of their hatchings have died from *"range paralysis"* at the age of 5 months. Shortly before the mortality started a dove(?) roosted with the flock for several weeks. At the time Mrs. B. became ill about 150 to 175 chickens were on the premises. Apparently, the mortality continued and by the middle of May only 31 chickens, mostly emaciated and partly paralyzed, could be secured for autopsies. Since no gross lesions of ornithosis were noted, the sera of 12 birds were tested in the agglutination test with *Microbacterium multiforme*. Two sera gave specific reactions in dilutions of 1:8++++ to 1:16+++ and one in a dilution of 1:2++++ to 1:4++. Three chickens (No. 1, No. 5, and No. 11) with negative serological findings yielded the ornithosis virus by repeated passage through mice. For technical

TABLE I.
Pathogenicity of Chicken-Ornithosis Virus.

| Bird | Mode of infection cc | Source of virus | Clinical and anatomical findings at time of death | Microscopic findings | C.F. |
|---|---|---|---|---|---|
| Pigeon 1 | IC.* 0.03 | MB‡ (approximately 100,000 M.L.D. per cc) | Paralysis, death 7th day, meningitis | ∞ MMO¶ | — |
| ,, 2 | ,, | ,, | ,, ,, 10th ,, ,, | ,, ,, | — |
| Dove 1 (Streptopelia) | ,, | ,, | ,, ,, 4th ,, ,, | ,, ,, | — |
| Dove 2 | ,, | ,, | ,, ,, ,, ,, ,, | ,, ,, | — |
| Ricebird 1 | IM† 0.3 | ,, | Typical course, death 10th day, splenic tumor, purulent exudate over pericardium | ,, ,, | — |
| ,, 2 | ,, | ,, | Same on 15th day | ,, ,, | — |
| Dove 3 | IM 0.5 | MS§ (approximately 1,000 M.L.D. per cc) | Sick, recovered and sacrificed 104th day | Negative, no passage | 1:256++++ |
| ,, 4 | ,, | ,, | Same | Negative but virus in spleen by passage | 1:256++++ |
| Pigeon 3 | ,, | ,, | Sick, death 11th day, slight exudate | Moderate number of MMO | — |
| ,, 4 | ,, | ,, | Sick, death 41st day, emaciated, exudates | ∞ MMO | >1:256 |
| Chicken 1 (6 weeks old) | ,, | MS (approximately 10,000 M.L.D. per cc) | Sick, anemia, emaciated, death 69th day, pericarditis | Negative, virus in spleen and kidney by passage | — |
| Chicken 2 | ,, | ,, | Sick but recovered, sacrificed 165th day | No virus by passage | — |
| ,, 3 | IC 0.5 | ,, | Paralyzed, death 26th day, meningitis | ∞ MMO | — |
| ,, 4 | ,, | ,, | ,, ,, 11th ,, ,, | ,, ,, | — |
| Ricebird 3 | IM 0.5 | ,, | Sick, death 8th day, typical lesions | ,, ,, | — |
| ,, 4 | ,, | ,, | ,, ,, ,, ,, ,, ,, | ,, ,, | — |
| Parrakeet 1 | ,, | ,, | ,, ,, 10th ,, ,, ,, | ,, ,, | — |
| ,, 2 | ,, | ,, | ,, ,, 19th ,, ,, ,, | ,, ,, | — |
| Pigeon 5 (C.F.O.) | IC 0.03 | ML‖ (approximately 100,000 M.L.D. per cc) | Paralyzed, death 3rd day, meningitis | ,, ,, | — |
| Pigeon 6 | ,, | ,, | ,, ,, 10th ,, ,, | ,, ,, | — |

*IC = Intracranial.
†IM = Intramuscular.
‡MB = Mouse brain.
§MS = Mouse spleen.
‖ML = Mouse lung.
¶MMO = *Microbacterium multiforme* ornithosis.
C.F. = Complement fixation reaction.

reasons, the nature of the paralysis has not been investigated, and thus it is impossible to state with any degree of certainty that the mass mortalities on the poultry ranch colloquially designated as "range paralysis" were in any way attributable to ornithosis. However, the evidence conclusively proves the existence of spontaneous latent infections with this virus in the flock, and inductively connects the barnyard fowl with human illness. The patient not only fed the chickens but she cleaned the eggs soiled with excreta and removed the entrails in dressing the carcasses. There is likewise suggestive evidence that the virus may have in an inapparent manner infected the other members of the family. The serum of the father (age 53), the daughter (27) and the oldest son (25) gave specific complement fixation reactions in dilutions of 1:4 to 1:8++++. That of the 7-year-old son was negative. Such reactions are significant since similar titers have been previously recorded in latent infections. In their broader implications to public health, these observations denote the existence of a large reservoir of psittacosis or ornithosis-like viruses in the bird kingdom. More than ever will it be the duty of the epidemiologist to study the ecology of the inhabitants of the barnyards and the pigeon lofts when he encounters a patient from which a meningo-pneumonitis-psittacosis-like virus has been isolated, or is being suspected on account of a positive serological reaction. Indirect transfer of virus with soiled feeds should not be overlooked. There is every reason to anticipate that as part of a broad evolutionary development, other birds will be found to be the hosts of an ornithosis parasite which has accompanied them through generations of their ascendency.

### 13616

## *In vitro* Response of Lymphocytes to Minimal Doses of X-Rays.*

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The minimal dose of X-ray that produces a significant change in activity has been investigated for several types of living cells. Las-

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nitzki and Lea[1] studied the inhibition of mitosis in hanging drop cultures of fibroblasts. The cultures were exposed to 20-2000 r. For some stages of mitosis (especially the telophase) 30 r caused a reduction of mitosis to 50% of the controls. Although no tables of data are given, from the graphs it is apparent that about 10 r produced a 10 to 15% reduction in the mitotic count. Bliss and Packard[2] worked out the dosage survival curve for Drosophila eggs exposed to a total dose of 61-500 r. A small decrease in hatchability of the eggs was caused by the low doses. Stenstrom, King and Henschel,[3] in a study covering a wide range of dosage, found that a dosage of 39 r of X-rays decreased the *in vitro* migration of the lymphocytes 8.8%.

The present investigation was undertaken to determine the smallest dose of X-ray that could produce a significant decrease of migration of lymphocytes in cultures of adult mesenteric lymph nodes.

The technic used in this study was that described previously by the authors (King,[4] Stenstrom and King,[5] and Stenstrom, King and Henschel[3]). In each series the rabbit lymph node fragments were carefully separated in sets of 3 according to size, shape and color. One fragment from each set of 3 was used in the control 1 group, one in the control 2 group and the other in the experimental group. In this way there were visually identical cultures in the 3 groups. As may be noted by comparing the average values for control 1 and control 2 in Tables I and II, such "matched fragments" produce reliable quantitative results.

The fragments were suspended in serum in Carrel $D_5$ flasks. The flasks were placed in a special incubator and exposed to the desired X-ray dosage at 37.5°C. The control flasks were protected by ¼ inch of lead plate. After radiation the fragments were washed with sterile tyrode and planted as individual cultures in one drop of rabbit plasma and 3 drops of chick embryo-rabbit serum extract. Before the individual cultures were incubated they were given a code number so that the person who did the measurements did not know which were control and which were experimental cultures.

The cultures were incubated for 24 hours at 37.5°C as lying drops. The migration rims were measured at 24 hours of incubation under

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[1] Lasnitzki, I., and Lea, D. E., *Brit. J. Radiol.*, 1940, **13** N.S., 149.

[2] Bliss, C. I., and Packard, C., *Am. J. Roent. and Rad. Therap.*, 1941, **46**, 400.

[3] Stenstrom, W. K., King, Joseph T., and Henschel, Austin F., in press.

[4] King, Joseph T., *Arch. f. Exp. Zellforsch.*, 1930, **9**, 341.

[5] Stenstrom, W. K., and King, Joseph T., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 597.

TABLE I.
7½ Seconds of Radiation at 155 r Per Minute.

| Series | No. of cultures | Avg migration controls 1 | Avg migration controls 2 | Avg migration experimentals |
|---|---|---|---|---|
| 1 | 30 | 155.5 | 160.5 | 150.0 |
| 2 | 30 | 150.0 | 152.0 | 147.5 |
| 3 | 30 | 172.5 | 168.5 | 164.5 |
| 4 | 30 | 143.0 | 144.5 | 137.0 |
| 5 | 30 | 141.5 | 132.0 | 117.0 |
| 6 | 30 | 145.0 | 141.0 | 137.0 |
| 7 | 30 | 124.5 | 120.0 | 117.0 |
| 8 | 30 | 133.0 | 130.0 | 128.0 |
| 9 | 30 | 112.5 | 114.0 | 104.0 |
| 10 | 30 | 126.5 | 131.5 | 128.5 |
|  | Avg | 140.4 | 139.4 | 133.0 |

low power (60×) with an eye piece micrometer (114 e.p.u=1 mm).

Radiation data: 200 kv; 30 ma; filter 2 mm AL; 50 cm distance; 155 r per minute; half layer value 0.28 mm cu.

The results are given in the tables.

With 7½ seconds of radiation the average absolute difference between the experimental and control 1 is 7.4 (standard error 2.14). Inhibition in terms of control 1 is thus 5.3%.

With 3¾ seconds radiation the average absolute difference in terms of control 1 is 3.0 (standard error 0.82). However, if one uses control 2 the results are unconvincing in spite of the fact that the absolute difference is slightly greater.

*Conclusion.* A dosage of approximately 20 r (7½ sec at 155 r/sec) produces a significant inhibition in the migration rate of lymphocytes. At one-half this dosage the results are of questionable significance.

TABLE II.
3¾ Seconds of Radiation at 155 r Per Minute.

| Series | No. of cultures | Avg migration controls 1 | Avg migration controls 2 | Avg migration experimentals |
|---|---|---|---|---|
| 1 | 30 | 167.5 | 164.0 | 163.0 |
| 2 | 30 | 187.0 | 198.5 | 186.5 |
| 3 | 30 | 135.0 | 139.5 | 130.0 |
| 4 | 30 | 135.5 | 136.5 | 131.5 |
| 5 | 30 | 145.5 | 143.5 | 145.0 |
| 6 | 30 | 170.5 | 163.0 | 167.0 |
|  | Avg | 156.8 | 157.5 | 153.8 |

### 13617

# A Liver Function Test for Small Laboratory Animals.

Albert O. Seeler and Samuel Kuna. (Introduced by H. Molitor.)

*From the Merck Institute for Therapeutic Research, Rahway, N. J.*

Following studies[1] on the pharmacological behavior of certain phthalein dyes, Rosenthal and White[2] introduced bromsulphalein as an agent for the study of hepatic function. This test has been and is widely used in both clinical and experimental medicine. While increased dye retention cannot be definitely correlated with specific disturbance of the various functions of the liver, it does indicate liver damage of some sort, the degree of dye retention giving a grossly quantitative estimation of the extent of the damage.

Although the standard bromsulphalein test is applicable to the larger laboratory animal, such as the dog or the rabbit, the relatively large quantities of blood required for the test prevent its use in small laboratory animals which in many experiments are those of choice. We have, therefore, modified the method for application to rats and mice. The technic is simple, rapid, and in the rat it can be repeated at daily intervals by using alternate saphenous veins.

The application of the test to small animals is made possible by the replacement of the customary pipettes and test tubes with glass-capillaries, a suggestion made to us by Dr. A. N. Richards. The pyrex glass capillaries are machine drawn to an outside diameter of 1.2 mm and an inside diameter of 0.9 mm and are so uniform that minute fluid volumes can be estimated quite accurately by measuring the length of the fluid column.

*Experimental Technic.* The rat is weighed, securely tied to a board, and 10 mg per kg of a 1.0% bromsulphalein solution is injected into the saphenous vein. Four minutes and 50 seconds following the injection, the tail, having just been dipped into warm water and dried, is clipped and two capillaries 9 cm in length are filled with blood, leaving about 2 cm free for sealing. The ends of the capillaries are flame-sealed and the tubes are immediately spun at 3000 rpm; usually 2 to 3 minutes suffice to separate the serum and cells. Breakage of the pyrex capillaries in the centrifuge at 3000 rpm is rare in our experience, but they do not withstand much higher speed.

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[1] Rosenthal, S. N., and White, E. C., *J. Pharm. and Exp. Therap.*, 1924, **24**, 365.

[2] Rosenthal, S. N., and White, E. C., *J. A. M. A.*, 1925, **84**, 112.

The serum containing portion of the first capillary is broken off and serum in excess of 20 mm of capillary length (approximately 0.014 cc) is expelled. A 10% solution of sodium hydroxide is drawn up into the capillary to a distance of 1 mm (approximately 0.0007 cc) and then both serum and hydroxide are blown onto a glass slide and thoroughly mixed. This solution is then drawn back into the capillary and is ready for direct comparison with the bromsulphalein standard. In making the reading the non-alkalized serum of the second capillary is placed behind the standard in a suitable comparator box.

The standards are made on the assumption that the blood volume of the rat is approximately 10% of the body weight. In setting up the standard a 1-20 dilution with alkali is made so that the alkali dilution occurring in the test is corrected for. The 100% standard is obtained by adding 1 cc of a 5% solution of bromsulphalein and 12½ cc of 10% sodium hydroxide to 249 cc of water. By appropriate dilutions a series of standards from 5 to 90% is prepared, drawn into capillaries and sealed. These standards, if protected from bright light will keep for several months.

In a series of 70 normal rats, the dye retention at the end of 5 minutes was found to be 10% or less in all but 5 of the animals. Two of the latter showed a 20% retention and the remaining 3 showed a 15% retention.

That the percentage retention readings can be duplicated on the same animal and that the dye in itself does not damage the liver is shown by the following experiment: Ten normal rats, all of whom showed 10% retention or less were given 10 mg per kg of bromsulphalein 7 times over a period of 2 weeks. At the end of this period all the animals still showed the same degree of dye retention as at the beginning of the experiment.

This test can be applied to mice by the same technic as described above for the rat. It is necessary, however, to give the mouse 25 mg of bromsulphalein per kg and the color standards have to be made up to conform with this dosage. Either the saphenous or the tail veins may be used for injection of the dye. The test is not as satisfactory in the mouse as it is in the rat as on occasion difficulty is experienced in separating the serum from the cells. Furthermore, the number of tests on an individual mouse is usually limited as the veins tend to thrombose following the injections.

In order to correlate the dye retention with the severity of liver damage, rats were subjected to various degrees of phosphorus poisoning. Table I gives the degree of dye retention in these poisoned

TABLE I.
Bromsulphalein Retention Following Phosphorous Poisoning.
10% dye retention before adminis. of P.

| Rat No. | Daily s.c. dose of P in sesame oil mg/100 g | No. of days | % dye retention after administr. of P | Liver damage estimated by histological study |
|---|---|---|---|---|
| 1 | .375 | 2 | 15 | 0 |
| 2 | .375 | 2 | 10 | 0 |
| 3 | .375 | 4 | 50 | +++ |
| 4 | .375 | 4 | 50 | +++ |
| 5 | .375 | 5 | 60 | ++++ |
| 6 | .375 | 5 | 45 | +++ |
| 7 | .75 | 2 | 20 | 0 |
| 8 | .75 | 2 | 15 | 0 |
| 9 | .75 | 3 | 40 | ++ |
| 10 | .75 | 3 | 20 | 0 |
| 11 | .75 | 4 | 45 | ++ |
| 12 | .75 | 4 | 60 | ++++ |

animals together with the amount of liver damage as judged by study of microscopic sections stained with hematoxylin-eosin, and Sudan IV. These data show a relation in the amount of phosphorus given to the degree of dye retention and to the liver damage as estimated histologically.

*Summary.* A simple and rapid technic for performing the Rosenthal-White bromsulphalein liver function test on rats and mice is described. The test gives a roughly quantitative estimate of the degree of liver damage and since it can be repeated on the same animal it is of use in following the progress or regression of liver disturbances.

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## 13618 P

## Current Control in Electroshock Therapy.*

MILTON S. PLESSET. (Introduced by C. A. G. Wiersma.)

*From the William G. Kerckhoff Laboratories of the Biological Sciences and the Norman Bridge Physics Laboratory, California Institute of Technology, Pasadena.*

Electroshock treatment consists in the passage of alternating current for a few tenths of a second through electrodes placed on the temples of the patient. The intensities of the current vary from 200 milliamperes to the neighborhood of 1,000 milliamperes. The customary procedure is to apply a certain voltage obtained from the

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* Aided by a grant from the Department of Institutions, State of California.

general alternating supply line. If the resistance of the head of the patient could be determined in advance, the current which would flow for a given voltage could be predicted. It is known that this is not the case. The patient's head resistance is variable and unpredictable. A test measurement of resistance with a small current gives high values (roughly from 500 to 5000 ohms) which are not correlated with the resistances actually offered to the large shock current. The resistances for the large shock currents also show appreciable variation, not only from patient to patient, but also in the same patient at different times. For electrodes of 15-25 cm$^2$ in area, these resistances vary from about 100 to 300 ohms. It is clear that the application of a given voltage to the electrodes will give fluctuating and uncontrollable values for the shock current (Table I). Of the two factors which determine the effect of the shock, the duration of the current, and the strength of the current, the first is readily controlled by means of a suitable timer; it is, however, just as essential to control the second factor. The physical conditions of the shock would then be fixed, and could be prescribed in advance.

An electroshock apparatus which gives a preassigned current to the patient regardless of the resistance has been designed and constructed in our laboratories, and is now in satisfactory operation at the Patton State Hospital, Patton, California. To obtain a constant current regardless of resistance requires alternating current regulation which may be secured by a variety of circuits. The method of regulation chosen for this apparatus consists in feed-back with low amplification

TABLE I.

| Patient | Test resistance, ohms | Shock voltage | Shock duration, seconds | Shock current, milli-amperes | Shock resistance, ohms |
|---|---|---|---|---|---|
| A | 700 | 100 | .3 | 420 | 240 |
| | 1350 | 100 | .5 | 450 | 220 |
| | 1700 | 100 | .5 | 350 | 290 |
| B | 250 | 90 | .2 | 830 | 110 |
| | 385 | 90 | .2 | 875 | 100 |
| | 440 | 100 | .2 | 745 | 135 |
| C | 725 | 125 | .3 | 900 | 140 |
| | 900 | 125 | .3 | 550 | 230 |
| | 1000 | 125 | .3 | 720 | 170 |
| D | 1600 | 120 | .5 | 450 | 270 |
| | 3000 | 130 | .5 | 780 | 170 |
| | 2500 | 130 | .5 | 650 | 200 |
| E | 850 | 100 | .3 | 400 | 250 |
| | 700 | 100 | .3 | 580 | 170 |

and phase inversion into a pair of 813 transmitter tubes in push-pull which are transformer coupled to the output circuit. A ballast resistor of 125 ohms is in the output circuit when the patient is not connected; the treat button transfers the current from this resistor to the patient for the duration of the shock. The power dissipation provided by the 813 tubes in this compensation circuit is 200 watts, so that the range of resistance compensation is ample for electroshock purposes. The apparatus in its present form gives currents from 120 to 1000 milliamperes. The operator presets the current and measures its intensity with a meter which reads continuously before, during, and after the shock. For the variations in head resistances found in electroshock, the current intensity variations are less than 1%. The durations of the shock available in this apparatus may be changed by steps of 0.1 second from 0.1 to 1.0 second. A test resistance measurement is still used, but only with the purpose of assuring that good contact is maintained through the electrodes to the head.

It may be remarked that even when the physical conditions of electroshock are accurately controlled, as they are in the apparatus here described, there may be variations in the physiological response of the patient. Nevertheless, accurate control of these physical conditions is certainly very desirable, and is clearly a prerequisite of any systematic study of electroshock itself.

Table I summarizes data on a few typical shocks given with an older electroshock apparatus of the non-regulating type constructed in our laboratories. The test resistance is measured with a small alternating current of 1 milliampere or less. The same electrodes (15 $cm^2$ in area) were used in all these shocks. A given shock voltage was applied and the resulting shock current was measured. The shock resistance is calculated from Ohm's Law: Shock resistance = shock voltage/shock current.

### 13619

## Protective Value of Antiviral Serum in Experimental Rabies Infection.

C. H. Yen.

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Recently Webster[1] has shown by animal protection test the inefficiency of most commercial rabies vaccines. Although the problem of finding a more satisfactory vaccine has attracted the attention of various investigators, it is as yet not fully solved. In view of this, it seems worthwhile to reëxamine the possible value of sero-prophylaxis of which contradictory results were reported by earlier workers.[2, 3, 4] While more recently Hoyt and coworkers[5] have demonstrated the protective action of antiviral serum in white mice, their results only showed an increase in the percentage of survivals over the untreated controls. Jonnesco[6] also has reported the prolongation of the incubation period of 3 out of 5 guinea pigs previously treated with antiviral serum. In the light of such suggestive but inconclusive results, it seems desirable to test more carefully the efficacy of antirabies serum with control of the important experimental factors such as weight and age of test animals, dosage of virus, route of infection, quantity of serum in antiviral units given, and the interval between infection and antiserum administration. In this communication observations indicating the beneficial effects of a single dose of antiviral serum against multiple fatal doses of rabies infection are presented.

*Materials and Method.* A strain of fixed rabies virus (Habel)* maintained by frequent intracerebral passages in mice was used. Antiviral sera were prepared from rabbits by repeated intraäbdominal inoculations of 5 cc doses of a phenol-treated vaccine given twice weekly for the first 4 weeks followed by similar injections with a live vaccine for 4-6 weeks. The live vaccine is a 10% brain emulsion of rabbit dying on 8-10th day of infection. The phenol-treated vaccine is a similar emulsion containing 0.5% phenol which has been incubated at 37°C for 48 hours. All vaccines were used within 2 weeks after preparation. The potency of antiviral serum is expressed in antiviral units. One antiviral unit is the minimal amount of serum contained in the 0.03 cc mixture of serum and virus which after incubating at 37°C for one hour, suffices to inactivate 1,000 intracerebral M.L.D. of the fixed virus for Swiss mice weighing 10-12 g (5-6 weeks old). One intracerebral M.L.D. is the minimum amount of virus given in 0.03 cc that will cause death of 50% of inoculated animals. One intramuscular M.L.D. is the minimal amount of virus in 0.1 cc which when injected into the left gastrocnemius is just sufficient to cause death of 50% of injected animals. Since the number of M.L.D. contained in the same virus suspension varies somewhat depending on the size of animals tested, we have employed animals of approximately the same age and weight for both control and test groups in each experiment. Sera containing $3.3 \times 10^4$ antiviral units were usually obtained and were kept in frozen state in the refrigerator without any preservative. Normal rabbit serum regularly showed no antiviral power.

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[1] Webster, L. T., *J. Exp. Med.*, 1939, **70**, 87.

[2] Fermi, C., *Centralbl. f. Bakt.*, 1909, **52**, 576.

[3] Kraus, R., and Holobut, T., *Z. f. Immunitätsforsch. u. exp. Therap.*, 1909, **3**, 130.

[4] Marie, A., *Ann. Inst. Pasteur*, 1908, **22**, 271.

[5] Hoyt, A., Fisk, R. T., and Tracy, R. L., *J. Inf. Dis.*, 1936, **59**, 152.

[6] Jonnesco, D., *Compt. rend. Soc. Biol.*, 1939, **130**, 1145.

* This strain of virus originally obtained from Dr. Habel, National Institute of Health, Washington, D.C., has been kindly supplied to us by the Squibb Institute for Medical Research, N.J.

*Effect of Antiviral Serum on Intracerebral Infection.* Normal Swiss mice weighing 15-16 g were divided into groups A. B. and C. 0.03 cc from each 10-fold dilution ($10^{-1}$ to $10^{-7}$) of the fixed virus suspension was given intracerebrally to 6 animals from each group. Within 10-15 minutes following the inoculation, each of the animals in group B received intraäbdominal injection of 0.5 cc normal rabbit serum diluted 1:3 in saline and those of group C, 0.5 cc of antiviral serum (1:3) containing $10^4$ antiviral units. Group A animals were not treated. These animals were then observed for a period of 6 weeks. Table I records day of death of these animals. From this, it is obvious that the amount of antiserum given exerted a favorable effect by showing survival of a few animals with marked prolongation of lives of all the rest of the treated animals as compared with the untreated controls or those treated with normal serum.

That the prolongation of the life-span is induced regardless of dosage of infection ranging from 10 to $10^6$ M.L.D. is difficult to explain. It is possible that the prolonged course of disease is caused by the small amount of virus which before coming in contact with antiserum, has already entered the nerve cells. The free virus remaining at the site of injection was probably inactivated by the absorbed antiviral serum.

TABLE I.
Effect of Antiviral Serum on Intracerebral Infection.

| Treatment | Virus Dilution | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1:10 | $1:10^2$ | $1:10^3$ | $1:10^4$ | $1:10^5$ | $1:10^6$ | $1:10^7$ |
| A Control | *5,5,5,<br>5.6.6 | 5,6,6,<br>6,7,7 | 6,6,6,<br>7,7,7 | 6,6.7,<br>7,8,9 | 7.7,7,<br>7,8,8 | 7,8.8,<br>8,8,9 | 10,11,S,<br>S,S,S |
| B Normal serum | 5,5,6,<br>6,6,6 | 6,6,7,<br>7,7,7 | 7,7,7,<br>7,7,7 | 7,7,7,<br>7,7,7 | 7,7,8,<br>8,8,8 | 8.9.9,<br>9.9,10 | 9,10,11,<br>S,S,S |
| C Immune serum | 11,11,16,<br>16,17,(S) | 10.13.14.<br>16.17,(S) | 12,13.13,<br>14.16,S | 9.12.13,<br>14,15,16 | (2),5,14,<br>14,16,(S) | 14.15.16,<br>16,17,S | — |

TABLE II.
Effect of Antiviral Serum on Intramuscular Infection.

| Treatment | Serum dilution | Antiviral units | Virus Dilution | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1:10 | 1:40 | 1:160 | 1:640 | 1:2.560 | 1:10,240 |
| I None | | 0 | 7.8.8,<br>8,8,9 | 8.8,8,<br>9,9,10 | 8,8,9,<br>9,9,10 | (1).8.9,<br>9.10.11 | 9.9.10.<br>10,10,11 | 11,12.S,<br>S,S,S |
| II Normal serum | 1:1 | 0 | 8,8,8,<br>9,9.9 | 8,8,8,<br>8,9.10 | 8.8,9,<br>9,10,10 | 8.8.8,<br>8.9.10 | (2),9.10,<br>10,10.10 | 11.12,14.<br>S,S,S |
| III Immune serum | 1:1 | $1.6\times10^4$ | (2),8,27,<br>(S),S,S | 12,(S),(S),<br>S,S,S | 22,S,S,<br>S,S,S | S,S,S,<br>S,S,S | S,S,S,<br>S,S,S | —<br>— |
| IV ” ” | 1:4 | $4\times10^3$ | 11,11,12,<br>22.26,27 | 15,18,(S),<br>S,S,S | 8,S,S,<br>S,S,S | S,S,S,<br>S,S,S | S,S.S,<br>S,S.S | —<br>— |
| V ” ” | 1:10 | $1.6\times10^3$ | 10,10,13,<br>13,14,(S) | 12,12,13,<br>16,17,(S) | 9.10,11,<br>14,S,S | 12,S,S,<br>S,S,S | S,S,S,<br>S,S,S | —<br>— |
| VI ” ” | 1:100 | $1.6\times10^2$ | — | — | 9,9,10,<br>10,12,14 | 9.10.17.<br>18,(S),S | 10,S,S.<br>S,S,S | S,S,S,<br>S,S,S |

*The numerals denote day of death following infection.
(S) Animals showing symptoms of paralysis but surviving over 6 weeks.
S Animals surviving over 6 weeks without showing symptoms.
(1) and (2) Animals dying from undetermined cause.

*Effect of Antiviral Serum on Intramuscular Infection.* Six groups of Swiss mice weighing 10-12 g were each given 0.1 cc of serial 4-fold dilutions of the fixed virus into the left gastrocnemius. Within 10-15 minutes after inoculation, 0.5 cc of undiluted normal rabbit serum was injected intraäbdominally to each of the animals in Group II, and similarly, 0.5 cc of antiserum undiluted, 1 :4, 1 :10, and 1 :100 in saline were separately given to animals in Groups III, IV, V, and VI respectively. Group I animals were left untreated. Table II summarizes observations on these animals. It will be seen that the antiviral serum diluted as high as 1 :100 ($1.6 \times 10^2$ antiviral units) was only able to protect a majority of the animals against 4 intramuscular M.L.D. But with the increase of the antiviral units given, protection against more intramuscular M.L.D. was obtained. The beneficial effect afforded by the antiserum against intramuscular infection is far better than against the intracerebral infection.

*Vaccine and Antiviral Serum on Intracerebral Infection.* Since in our hands, vaccination of animals with phenol-treated vaccine according to Habel's[7] procedure of potency test gave only partial protection for animals infected with highly fatal doses, the following experiment was carried out to determine the combined effect of the antiviral serum and vaccine treatment. Animals weighing 10-12 g were divided into 2 groups, vaccinated and unvaccinated, of 18 animals each. In the vaccinated group, all the animals were given intra-abdominal injections of 0.25 cc of a 0.5% phenol-treated vaccine on alternate days for 6 injections. Fourteen days after the first injection, all the animals in both groups were each given intracerebrally 0.03 cc (100,000 intracerebral M.L.D.) of the virus. Immediately following virus inoculation, 0.5 cc of 1 :3 normal rabbit serum was given intraäbdominally to 6 animals from both groups, and 0.5 cc of antiserum 1:3 ($5 \times 10^3$ antiviral units) was given separately to another 6 animals from both groups. The remaining 6 animals from both groups were left untreated. Table III compares the results observed with normal serum- and immune serum-treated animals. The untreated controls showed no essential difference from the normal serum treated groups, and are not tabulated. This table clearly shows that whereas animals receiving vaccine and normal serum gave only 50% survival, those receiving vaccine and antiserum all survived over 6 weeks without showing symptoms. The unvaccinated animals receiving antiserum alone showed prolongation of the life-span of animals, confirming the results obtained above. The

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[7] Habel, K., *Public Health Reports*, **1940**, **55**, **1473**.

TABLE III.
Effect of Vaccine and Antiviral Serum on Intracerebral Infection.

| | Vaccinated | Non-vaccinated |
|---|---|---|
| Normal serum | 8,8,11,S,S,S | 6,6,6,7,7,7 |
| Immune serum | S,S,S,S,S,S | 10,13,15,15,16,17 |

satisfactory protection afforded by the combined vaccine and antiserum treatment as compared with the administration of either alone is of interest. Further studies along this line are in progress to explore in greater detail its practicability.

*Conclusion.* Administration of a single dose of potent antiviral serum intraäbdominally 10-15 minutes after infection is capable of prolonging the life-span of the intracerebrally infected animals, and completely saving the lives of some of the intramuscularly infected animals depending on the dosage of infection and amount of antiviral units given. The combined use of vaccine and antiserum afforded full protection to the animal against 100,000 intracerebral M.L.D. of the virus.

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### 13620

## Effect of Malonate and Iodoacetate on Respiration of Brains of Rats of Various Ages.

David B. Tyler.*

*From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, California.*

It has been shown that the oxygen uptake of the excised brain of the infant rat is much lower than that found in the adult.[1, 2] Furthermore, during the development of the brain of this animal 3 levels in its metabolism can be distinguished.[3] The lowest level is found during the first week of life; the highest occurs between the fourth and seventh week; the third, or adult level, is reached at about 20 weeks, and is significantly lower than the second level. Parallel changes are found to occur also in the rate of glucose utilization. In contrast to this, the glycolytic activity during the first week is relatively very high, but falls off rapidly, so that at 31 days it is of the same order as that of the adult.[3]

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* Hixon Fund Fellow.

[1] Himwich, H. E., Baker, Z., and Fazekas, J. F., *Am. J. Physiol.*, 1939, **126**, 601.

[2] Tyler, D. B., and van Harreveld, A., *Am. J. Physiol.*, 1941, **133**, 472.

[3] Tyler, D. B., and van Harreveld, A., in press.

This report, the first of a series concerned with enzymatic activity during the development of the brain, deals with the degree of inhibition produced by malonate and by iodoacetate on the oxygen uptake of brains of rats of various ages.

*Methods.* Oxygen consumption was measured in Warburg Respirometers using Ringer's-glucose media buffered with phosphate to pH 7.4.[4] The rats were killed by decapitation, the brains quickly removed, and the pallium (cerebral hemisphere minus the corpora striata and olfactory lobes) was separated from the neuraxis. One hemisphere of each animal served as a control, and was minced quickly and suspended in tared vessels containing the media without the inhibitor. The hemisphere of the other side was likewise minced and placed in vessels containing the inhibitor. The mince was distributed in vessels so that approximately the same weight of tissue was in each (150 mg ±20). This necessitated the pooling of several litter mates in the case of newborn rats. The vessels were quickly weighed and placed in the bath at 38°C. This entire procedure—killing, preparing the tissue, weighing the vessels containing the mince, washing the manometers with oxygen, and placing them in the bath, took from 30 to 35 minutes (for 6 vessels). This allowed 15 to 20 minutes for the manometers to reach equilibrium before the initial readings at 50 minutes were taken. In this report, the oxygen consumption found between 90 and 110 minutes after the animal was killed, and expressed as $mm^3$ of $O_2$ consumed/g of wet weight of tissue/hour, was used. For comparative results, this procedure has been found to be satisfactory as the oxygen utilization for a given region of the brain of rats of the same age is very constant.

*Results.* It can readily be seen from Table I that the brain of a newborn rat is comparatively insensitive to malonate. However, an increased sensitivity develops very quickly, so that at 10 days of age the percentage of inhibition is of the same order as that found in the adult. A point of special interest is the residual oxygen uptake found in the malonate exposed tissues. Despite the fact that there is a steady increase in percentage of inhibition during the first 10 days, the residual respiration remains constant at about 550 $mm^3$/g of wet weight of tissue/hour. Between the tenth and sixteenth day, there occurs an increase in this residual respiration that amounts to 260 $mm^3$ at 16 days, 410 $mm^3$ at 31 days, and 300 $mm^3$ in the adult.

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[4] Dixon, M., *Manometric Methods*, The University Press, Cambridge, 1934.

TABLE I.

| Age (days) | Control | 0.01 M Malonate* | % Inhibition | Age (days) | Control | 0.01 M Malonate* | % Inhibition |
|---|---|---|---|---|---|---|---|
| 1 | 656 | 547 | 16.9 | 10 | 1080 | 530 | 50.9 |
| 2 | 582 | 507 | 12.9 | 16 | 1500 | 810 | 46.0 |
| 6 | 814 | 590 | 27.6 | 31 | 1900 | 960 | 49.5 |
| 8 | 712 | 559 | 34.3 | Adult | 1723 | 864 | 50.0 |

*The final concentration was made up in Ringer-glucose-phosphate media.

The results with iodoacetate ($10^{-4}$ M) show that the brain of the newborn is very sensitive to this inhibitor. The oxygen uptake is reduced 46% in one-day-old rats, as compared with 26% in the adults. Thus, although the newborn is relatively insensitive to malonate, it is extremely sensitive to iodoacetate.

*Discussion and Conclusions.* Malonate has been assumed to be a specific inhibitor of succino-dehydrogenase.[5] If this assumption is correct, the above results indicate that the brain of the newborn does not depend on the oxidation of succinate to the same extent as does the adult's brain. However, the specificity of the action of malonate has been questioned, and evidence indicates that its effects are more complex and cannot be due primarily to an inhibition of that one enzyme.[6, 7, 8] Irrespective of which enzyme systems are inhibited by malonate, it is clear that, compared with the infant brain, the adult's is more sensitive to this substance. It is interesting to note that the increase in the respiration of the malonate poisoned tissue occurs at about the same time that myelinization is known to begin in the rat.[9, 10] Apparently, this increase is due to a system not very sensitive to malonate.

The results with iodoacetate seem to imply that the brain of the newborn depends, for its aerobic metabolism, on some of the degradation products of glycolysis to a relatively greater degree than does the brain of the adult.

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[5] Quastel, J. H., and Whetham, M. D., *Biochem. J.*, 1925, **19**, 525.

[6] Weil-Malherbe, H., *Biochem. J.*, 1937, **31**, 299.

[7] Greville, G. D., *Biochem. J.*, 1936, **30**, 877.

[8] Baumann, C. A., and Stare, F. J., *J. Biol. Chem.*, 1940, **133**, 183.

[9] Koch, W., and Koch, M., *J. Biol. Chem.*, 1913, **15**, 423.

[10] Sugita, N.J., *Comp. Neur.*, 1918, **29**, 1, 11, 241.

## 13621

# Changes in the Form of Inositol During Incubation of Eggs.

D. W. Woolley.* (Introduced by D. D. Van Slyke.)

*From the Laboratories of the Rockefeller Institute for Medical Research.*

Snell and Quarles[1] have reported recently that the content of inositol in eggs increases about 6 times during the period of incubation. Since the embryo was apparently able to synthesize this vitamin, it was suggested that the more mature organism was also possessed of this power, and hence that inositol was not a dietary essential for chicks. Analyses were performed on aqueous extracts of embryos which had been allowed to autolyze for short periods. Recently it has been demonstrated that at least 4 forms of inositol occur in tissues, *viz.*, free inositol, inositol hexaphosphate,[2] a new phosphatide,[3, 4] and a water-soluble, non-dialyzable substance.[5, 6] It has also been found that yeast responds only to free inositol.[7] Since several of the combined forms of inositol are not split by autolysis and since the response of yeast was the basis of the method of analysis used by Snell and Quarles, it seemed possible that what they had observed was a conversion of combined inositol to the free substance during incubation of eggs. It was, therefore, of interest to reinvestigate the problem with the aid of an analytical method which determines total inositol as well as, but separate from, free inositol.[6, 8] Total inositol was estimated by the method of Woolley[8] and free inositol by the modification recently described.[6] Fertile eggs were obtained from commercial sources (not always the same). They were incubated at 38.9°C and 70% humidity in a standard incubator. All analyses were based on the weights of the eggs at the beginning of the period of incubation. Before an egg was taken for analysis it was candled in order to assure that only living embryos were examined. Three separate experiments were performed. In the first, groups of 2 eggs were analyzed for free and total inositol at 0 and 20 days,

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[*] With the technical assistance of A. G. C. White.

[1] Snell, E. E., and Quarles, E., *J. Nutrition*, 1941, **22**, 483.

[2] Rapoport, S., *J. Biol. Chem.*, 1940, **135**, 403.

[3] Klenk, E., and Sakai, R., *Z. physiol. Chem.*, 1939, **258**, 33.

[4] Folch, J., and Woolley, D. W., *J. Biol. Chem.*, 1942, **142**, 963.

[5] Woolley, D. W., *J. Biol. Chem.*, 1941, **139**, 29.

[6] Woolley, D. W., *J. Exp. Med.*, 1942, **75**, 277.

[7] Woolley, D. W., *J. Biol. Chem.*, 1941, **140**, 461.

[8] Woolley, D. W., *J. Biol. Chem.*, 1941, **140**, 453.

TABLE I.
Average Inositol Content of Eggs at Various Stages of Embryonic Development. Numbers in parentheses indicate maximal deviation from the mean.

| Incubation, days | No. of eggs | Avg inositol content, μg/mg |  |
|---|---|---|---|
|  |  | Total | Free |
| 0 | 11 | .22 (± .05) |  |
|  | 18 |  | .046 (± .01) |
| 7 | 2 | .24 (± .0 ) |  |
| 10 | 2 | .21 (± .04) |  |
| 14 | 2 | .26 (± .04) |  |
| 20 | 10 | .24 (± .03) |  |
|  | 8 |  | .19 (± .05) |

and for total inositol at 7 and 14 days of incubation. In the second, the eggs were taken after 0, 10, and 20 days of incubation. In the third, groups of 6 eggs were analyzed at 0 and 20 days of incubation.

The average content of inositol in an egg at various periods of incubation is shown in Table I. The values for free inositol at the beginning and end of the incubation period are also shown. It can be seen that the total inositol in an egg did not increase significantly during the period of embryonic development. However, the proportion which is free increased. All of the inositol in aqueous extracts of eggs before incubation was free, and hence it was concluded that the combined inositol in such eggs was present as one of the water-insoluble forms. The fact that nearly all of the inositol in a hatching chick is free is of interest when it is recalled that in many tissues only about half of the vitamin is in the free state.[6]

*Summary.* The total content of inositol in a hen's egg does not increase during incubation, but the proportion of the total amount which is free and extractable by water does increase.

### 13622

## Glycine Requirement of the Chick.

H. J. Almquist and E. Mecchi.

*From the Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley.*

It has been shown that glycine is required in the diet of the young chick for an optimum rate of growth.[1, 2, 3] Experiments have been

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[1] Almquist, H. J., Stokstad, E. L. R., Mecchi, E., and Manning, P. D. V., *J. Biol. Chem.*, 1940, **134**, 213.

continued in an effort to obtain an estimate of the amount of glycine needed for this purpose. The diet used in these studies, except for the sources of protein to be mentioned, has been previously described in detail.[4]

A series of diets were made up containing 25% casein which had been washed with dilute acetic acid and with water, then dried in a current of air at 30°C. ½% of arginine monohydrochloride, and varying levels of synthetic glycine were also added. S. C. White Leghorn chicks were reared for 7 to 10 days on a practical diet, then carefully selected for uniform weight and condition, divided into groups of the same average weight and given the experimental diets. Rate of growth was determined by individual daily weights. Results obtained with the casein diets are given in Table I.

The results in Table I contain several features of interest. When no added glycine was present in the diet, the growth rate of chicks was distinctly low. Glycine added at levels of 0.5 and 1.0% did not result in optimum rates of gain. This optimum was attained in both series at 1.5% of added glycine. On the other hand, 2.0% glycine seemed to be harmful in that the rate of gain was distinctly lowered. There have been indications previously that 2% or more of uncombined glycine might be detrimental to chicks.[1]

Although receiving less than 1/10 the glycine given the most rapidly growing group, the basal group attained in each case approxi-

TABLE I.
Growth-Promoting Effects of Glycine in Chick Diets.

| | | Exp. 1* Avg gain per chick per day | | Exp.2† Avg gain per chick per day | |
|---|---|---|---|---|---|
| Supplement | Level % | g | % | g | % |
| None | — | 3.9 | 4.9 | 4.3 | 5.2 |
| Glycine | 0.5 | 4.4 | 5.2 | — | — |
| ” | 1.0 | 4.9 | 5.9 | 5.5 | 6.3 |
| ” | 1.5 | 5.9 | 6.6 | 5.9 | 6.8 |
| ” | 2.0 | — | — | 4.9 | 5.9 |
| Glycylglycine | 1.0 | — | — | 6.0 | 6.8 |

*At the start of the experiment each group contained 5 chicks with an average weight of 61 g. The experiment lasted 11 days. The percentage gain is calculated on the average of initial and final weights.

†At the start of the experiment each group contained 6 chicks with an average weight of 51 g. The experiment lasted 10 days.

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[2] Almquist, H. J., and Mecchi, E., *J. Biol. Chem.*, 1940, **135**, 355.

[3] Hegsted, D. M., Hier, S. W., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **139**, 863.

[4] Almquist, H. J., and Mecchi, E., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 526.

TABLE II.
Rates of Gain of Chicks in Relation to Combined Glycine Content of Diet.

| Protein or glycine source | Level % | Calc.* glycine content of diet % | Avg gain per chick per day % |
|---|---|---|---|
| Edestin<br>Casein | 25<br>5 | 1 | 6.9 |
| Edestin<br>Casein<br>Glycine | 20<br>10<br>0.2 | 1 | 7.0 |
| Casein<br>Gelatin | 20<br>10 | 2.6 | 6.8 |
| Casein<br>Gelatin | 25<br>6 | 1.6 | 6.9 |
| Casein<br>Gelatin | 25<br>4 | 1.1 | 6.8 |
| Arachin<br>Gelatin | 25<br>5 | 1.3 | 6.9 |

*Based upon summarized data for proteins as given by Calvery, H. O., *Chemistry of the Amino Acids and Proteins* (edited by C. L. A. Schmidt), Charles C. Thomas, publisher, Baltimore, Maryland, first ed., p. 217.

mately ¾ of the optimum rate of gain. This suggests that the chick has a limited ability to synthesize glycine; however, a critical test of such ability must await studies with glycine-free diets. Evidence for the synthesis of glycine by chick embryos has been reported.[5]

Glycine fed in combination may be somewhat more effectively used as compared to free glycine. For example, glycylglycine at a level of 1% (equivalent to 1.1% glycine) was as effective in growth promotion as free glycine at 1.5%. Other dietary combinations of proteins that have been tested in similar amino acid studies give further evidence that 1% of glycine in protein combination is adequate for an optimum rate of gain. (Table II.) While these results were not obtained in the same series of tests, they represent maximum rates of growth on diets and under conditions that were otherwise similar.

*Summary.* The glycine requirement of the young chick for optimum rate of growth is approximately 1.5% of the diet when provided as free glycine, and 1.0% when provided in combination as in gelatin, edestin, casein and glycylglycine. The young chick probably has a limited ability to synthesize glycine.

The authors are indebted to Merck and Co., Inc., for donation of the arginine monohydrochloride used in this work.

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[5] Patton, A. R., and Palmer, L. S., *J. Nutrition,* 1936, **11**, 129; Patton, A. R., *J. Nutrition,* 1937, **13**, 123.

## 13623 P

# Magnesium Requirement of the Chick.

H. J. Almquist.

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During studies on simplified rations for chicks it became evident that one deficiency in the rations was of a mineral nature. The deficient inorganic growth-promoting substance was subsequently identified as magnesium. As a result of these studies it has been shown that the magnesium requirement of the chick is comparatively high, sufficiently so as to constitute a very influential factor in experimental chick diets. The purified diet used in this work has been described elsewhere.[1] Without a magnesium supplement, the diet contained approximately 40 p.p.m. of magnesium.

Four quantitative experiments have been conducted. Magnesium was added to the diet as the sulphate (Mg content determined by analysis) to provide various levels of magnesium up to 600 p.p.m. The growth rates of chicks fed diets containing added magnesium at 120, 240, and 250 p.p.m. were in every case lower than the growth rates at 350, 450, 480, and 600 p.p.m. 350 p.p.m. may have been a barely adequate supplementary level. The growth rates at 350 or more p.p.m. were equivalent to those of chicks fed a practical rearing ration. Representative data from one experiment started with 7-day-old chicks are cited in Table I.

On the basal diet chicks grew slowly for approximately 1 week, then ceased growing and became lethargic. When disturbed, these chicks frequently passed into a brief convulsive, gasping, and finally a

TABLE I.
Comparative Weights of Chicks Reared on Diets Supplemented with Magnesium Sulphate.

| No. of chicks started | Avg wt at start, g | Mortality No. | Avg wt after 17 days on diet, g | Mg added to diet, p.p.m. |
|---|---|---|---|---|
| 10 | 56.4 | 7 | 80 | 0 |
| 10 | 56.2 | 0 | 136 | 250 |
| 10 | 56.2 | 0 | 157 | 350 |
| 10 | 56.4 | 0 | 158 | 450 |
| 10* | 56.1 | 0 | 161 | — |

*Reared on a practical diet throughout.

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[1] Almquist, H. J., and Mecchi, E., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 526.

comatose state which sometimes terminated fatally, but usually ceased in a few minutes. Such tetanic symptoms are well known for other species in magnesium deficiency.

The minimum magnesium requirement of the normal rat is approximately 50 p.p.m. in the diet.[2] The requirement of the chick appears to be much higher, approximately 400 p.p.m. during the first weeks of life. The response of the chick to magnesium deficiency is also much more rapid than that of the rat. In view of these results, it seems appropriate to advise adequate provision for the magnesium requirement in experimental chick diets.

### 13624 P

## Relationship of Agents of Trachoma and Inclusion Conjunctivitis to Those of Lymphogranuloma-Psittacosis Group.

GEOFFREY RAKE, MORRIS F. SHAFFER AND PHILLIPS THYGESON.

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While the nature of the etiologic agents of trachoma and inclusion blennorrhea is still not indisputably established, they appear to belong to the group of agents generally known as viruses and the great preponderance of evidence points to a causal relationship for the elementary and initial bodies which are characteristically present in the lesions.[1]

That these elementary and initial bodies, and other related structures are similar in morphology and dimension to those found in psittacosis and lymphogranuloma venereum has been pointed out.[2, 3] Moreover, with the exception of the glycogen reaction, which is positive for the large plaques in trachoma and inclusion blennorrhea,[4] but negative for those in psittacosis and lymphogranuloma venereum,[3] the tinctorial characteristics of the morphological units in all 4 diseases are similar.

A further bond between the two groups lies in the fact that of all the so-called virus diseases only 4, namely trachoma, inclusion blennorrhea, lymphogranuloma venereum, pneumonitis of mice,[5] together with the rickettsial infection of heart-water in sheep,[6] are known to respond to chemotherapy with the sulfonamide drugs.

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[2] Tufts, E. V., and Greenberg, D. M., *J. Biol. Chem.*, 1938, **122**, 715.

[1] Julianelle, L. A., *The Etiology of Trachoma*, The Commonwealth Fund, N.Y., 1938.

[2] Thygeson, P., *Am. J. Path.*, 1938, **14**, 455.

[3] Rake, G., and Jones, H. P., *J. Exp. Med.*, 1942, **75**, 323.

[4] Rice, C. E., *Am. J. Ophth.*, 1936, **19**, 1.

It has recently been demonstrated that a powerful complement fixing antigen can be prepared for lymphogranuloma venereum by the propagation of the agent of this disease in the yolk-sac of the embryonated chick egg.[7] Furthermore, it has been shown that this antigen gives marked cross fixation with sera from individuals infected with other members of this group of agents, *i. e.*, psittacosis or pneumonitis.[8] It seemed of interest, therefore, to test the sera of individuals infected with trachoma or inclusion blennorrhea by the same method to see whether any cross-fixation would occur here.

The results are shown in Table I. It was found that in the case of adults suffering from chronic trachoma (Institution EI) our usual routine of fixation at 37°C for 1½ hours gave fixation at serum titers comparable to those obtained with some lymphogranulomatous sera. Fixation was obtained not only with elementary body suspensions, but also with Seitz filtrates containing soluble antigen. The titers could be increased without loss of specificity by additional fixation in the icebox overnight before adding the sensitized sheep cells. This latter method was therefore adopted in the case of sera obtained from an outbreak of acute trachoma among male inmates of one cottage in an institution (L) for feeble minded, and by this method several such sera, *i. e.*, 6 of 8, were found to show fixation in low dilution. Frei tests were negative in those cases in which they were carried out.

One serum from an adult with inclusion conjunctivitis also gave fixation titers comparable to those of the trachoma patients, while the serum of an infant (G.D.) with acute inclusion blennorrhea showed evidence of slight fixing power.

These results indicate that the agents of both trachoma and inclusion conjunctivitis show a definite antigenic relationship to the Lymphogranuloma-Psittacosis group of agents. It seems to us possible that the low titers of fixation encountered may be due to less antigenic relationship than exists between lymphogranuloma

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[5] Rake, G., Jones, H. P., and Nigg, C., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, in press.

[6] Neitz, W. O., *J. S. African Vet. Med. Assn.*, 1940, **11**, 15.

[7] McKee, C. M., Rake, G., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 410.

[8] Rake, G., Eaton, M. D., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 528.

TABLE I.
Results of Complement Fixation Tests with Lymphogranuloma Venereum Antigens and Sera from Cases of Trachoma or Inclusion Conjunctivitis.

| | | Eye infection | | Serum titer in Comp. Fix. tests† vs. Lg. V. | | |
|---|---|---|---|---|---|---|
| Patient | Institution | Stage‡ | Duration | Elementary bodies | Soluble antigen | Frei test |
| Ga | E.I. | III | >10 yr. | 6 | | |
| E.B. | ” | IIa | 6 mo.? | 60 | 30 | Neg. |
| M.St. | ” | III | > 5 yr. | 30 | 15 | |
| L.Z. | ” | III | > 6 ” | 15 | 6 | |
| R.R. | ” | III | > 5 ” | < 6 | 15 | |
| L.C. | ” | III | >10 ” | 30 | 15 | |
| D'A. | ” | III | >15 ” | 15 | 30 | |
| F.S. | ” | I | | < 6 | 6 | |
| Y.L.S. | ” | IV | | 6 | — | Neg. |
| S.U. | ” | IV | | 6 | — | ” |
| M.T. | ” | IV | | 15 | — | ” |
| We. | ” | IV | | 15 | — | ” |
| V.B. | L* | I | | 6 | 6 | ” |
| J.C. | ” | III | | < 6 | 6 | ” |
| N.G. | ” | IIa | | < 6 | < 6 | ” |
| J.L. | ” | III | | <15 | 15 | ” |
| C.M. | ” | III | | < 6 | < 6 | ” |
| E.P. | ” | III | | 6 | 15 | ” |
| S.S. | ” | III | | 15 | 6 | ” |
| H.S. | ” | IIa | | 15 | 15 | ” |
| St.V. | E.I. | Inclusion conjunct. | 3 wk. | 60 | — | ” |
| G.D. | ” | ” ” | 2 ” | 6 | 6 | |

*The serum from 6 other feeble-minded trachomatous patients in the same cottage in institution "L" were tested but were either anticomplementary or gave non-specific fixation with the antigen controls prepared from normal yolk-sac.

†Fixation 1½ hours at 37°C + overnight at 2°C.

‡MacCallan's classification: Stage I, incipient trachoma, stage II established trachoma, IIa follicular hypertrophy predominant, IIb papillary hypertrophy predominant, III cicatricial trachoma, IV arrested trachoma.

venereum and psittacosis. On the other hand, it may be due to the local and limited nature of the disease which, because it has no phase of systemic invasion, builds up antibodies only slowly. In the acute cases of trachoma the short duration of the disease might also account for the slight or negative antibody response as might the physiological immaturity which existed in all of this latter group.

### 13625

## Sensitization to Horse Serum by Means of Adjuvants.

JULES FREUND AND KATHERINE MCDERMOTT.

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Lewis and Loomis[1] discovered that tuberculous guinea pigs produce more antibodies when injected with various antigens not related to tubercle bacilli than non-tuberculous guinea pigs. Dienes and Schoenheit[2] made the interesting observation that when egg white or horse serum is injected into tuberculous foci sensitization to these antigens is different from that usually seen in guinea pigs free of tuberculosis. Some aspects of the difference are: In tuberculous guinea pigs the cutaneous reactions to animal proteins appear and disappear slower; in tuberculous guinea pigs the reactions are often necrotic but in the non-tuberculous pigs very rarely if ever. This observation was confirmed by Freund[3] and by Hanks.[4] Dienes[5] reported that he was able to reproduce his experiment when killed tubercle bacilli were substituted for living ones. Three different methods of sensitization were partly or wholly successful. The first technic requires repeated injections of killed tubercle bacilli into a lymph node and after a lapse of a few weeks the repeated injections of a mixture of killed tubercle bacilli and egg white into the site of previous injections. The second method consists of two intraäbdominal injections. Twenty mg of killed tubercle bacilli are injected into the peritoneal cavity and 10 days later 20 mg of tubercle bacilli mixed with egg white. Some of the guinea pigs so treated die after the second injection from shock. The third method, which uses the testicles for the site of injections, does not yield consistent results.

In view of the advantages of employing killed tubercle bacilli instead of living ones we attempted to sensitize guinea pigs with killed tubercle bacilli and egg white. We made a single injection of killed tubercle bacilli into the subcutaneous tissue of the groin and 2 days later we injected egg white into the same site. The sensitization to egg white was not of the tuberculin type.

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[1] Lewis, P. A., and Loomis, D., *J. Exp. Med.*, 1924, **40**, 503; 1925, **41**, 327; 1926, **43**, 263.

[2] Dienes, L., and Schoenheit, E. W., *Am. Rev. Tub.*, 1929, **20**, 92; *J. Immunol.*, 1930, **19**, 41.

[3] Freund, J., *J. Exp. Med.*, 1934, **60**, 669.

[4] Hanks, J. H., *J. Immunol.*, 1935, **28**, 105.

[5] Dienes, L., *J. Immunol.*, 1928, **15**, 153; 1929, **18**, 531.

Since the addition of paraffin oil to killed tubercle bacilli enhances both the lesion and sensitization to tuberculin produced by killed tubercle bacilli[6] we[7] injected egg white into lesions produced by killed tubercle bacilli suspended in paraffin oil. Sensitization to egg white was not different from that observed in control guinea pigs. Landsteiner and Chase,[8] however, found that "sensitization to a simple chemical, picryl chloride, can be satisfactorily attained by means of intraperitoneal injections of the compound when killed tubercle bacilli suspended in paraffin oil were used as an adjuvant." These authors[9] later, using the same adjuvant, produced sensitization of the contact-type to conjugates made with homologous erythrocytic stromata. In the experiments just mentioned Landsteiner and Chase injected into the peritoneal cavity killed tubercle bacilli suspended in oil once or twice and soon afterwards the sensitizing substance.

It occurred to us that the difficulty of influencing sensitization to proteins (like egg white) by means of killed tubercle bacilli suspended in paraffin oil might be overcome by combining the antigen in the aqueous phase with a lanolin-like substance and in turn suspending the combination in paraffin oil containing tubercle bacilli. We used a material sold under the name Aquaphor* which can be combined with larger amounts of water than lanolin.

Aquaphor and heavy paraffin oil were sterilized in the autoclave. Tubercle bacilli of human type, a strain designated Jamaica No. 22, were grown on glycerol broth and heated in the Arnold sterilizer at 100°C for half an hour. They were dried *in vacuo* over phosphorus pentoxide, weighed and suspended in paraffin oil.

Horse serum was chosen for these experiments and was combined with the adjuvants in the following way. Ten ml of horse serum were added in small amounts to 10 ml of Aquaphor in a mortar and the two blended thoroughly. Twenty ml paraffin oil containing 40 mg killed tubercle bacilli were mixed in the mortar with the combination of horse serum and Aquaphor. The whole was shaken in a test tube and 0.5 ml injected into guinea pigs. One dose contained 0.125 ml horse serum, 0.125 ml Aquaphor, 0.5 mg killed tubercle bacilli and 0.25 mg paraffin oil. The guinea pigs weighed from 690 to 920 g. Five animals were injected intramuscularly into the back of the neck about 1 cm laterally from the midline[†] and 5 intramuscularly into the thigh. They were tested by intracutaneous injection of various concentrations of horse serum 7, 13, and 19 days later and again 6 and 12 months after injection. Another 10 guinea pigs were injected with 0.125 ml of horse serum diluted with saline solution into the back of the neck. They were tested with horse serum in exactly the same way as the animals sensitized with horse serum and the adjuvant 1, 2, 3 weeks and one year after sensitization. The cutaneous reactions were observed 3 to 4 hours after the injection and then every day until they disappeared. The experiment was repeated; 6 guinea pigs were injected with horse serum and the adjuvants into the neck and 6 with horse serum alone.

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[6] Freund, J., Casals-Ariet, J., and Hosmer, E. P., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 509; Freund, J., Casals-Ariet, J., and Genghof, D. S., *J. Immunol.*, 1940, **38**, 67.

[7] Freund, J., and Casals-Ariet, J., unpublished experiment, 1937.

[8] Landsteiner, K., and Chase, M. W., *J. Exp. Med.*, 1940, **71**, 237.

[9] Landsteiner, K., and Chase, M. W., *J. Exp. Med.*, 1941, **73**, 431.

* "Eucerin or Aquaphor, is an ointment base obtained from wool fat, possessing a much greater water-absorbing capacity than lanolin; it being capable of taking up 7 to 8 times its own weight in water. The saponifiable fatty acids are removed from the crude suint, from the cholesterin mixture (or soft fat) consisting of the esters of isocholesterol and oxycholesterol with oleic, myristinic and carnaubic acids. To this soft fat, petrolatum hydrocarbons are added as diluents and the product is marketed under the trade name." Arny, H. V., and Fischelis, R. P., *Principles of Pharmacy*, W. B. Saunders Co., Philadelphia and London, 1927, page 140.

The adjuvants modified sensitization in several respects. (1) The rate of appearance of the reactions to undiluted horse serum was similar in all groups of pigs, *i. e.,* conspicuous swelling was present in 3 hours; but in the first experiment with the higher dilutions of horse serum there was a difference in the rate of appearance of reactions. In the group with tubercle bacilli the swelling was noticeable only one day after the injection except in the test one year after the sensitiza-

TABLE I.
Necrotic Reactions to Horse Serum.

| | Guinea pigs sensitized | | | | | |
|---|---|---|---|---|---|---|
| | with horse serum + adjuvants | | | with horse serum alone | | |
| Time between sensitizing and tests | 1:1* | 1:10 | 1:100 | 1:1 | 1:10 | 1:100 |
| 1 wk. | — | 2/10 | 0/10 | 0/8 | 0/10 | 0/10 |
| 2 " | 8/10† | 4/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| 3 " | 5/8 | 0/8 | 0/8 | 0/10 | 0/10 | 0/10 |
| 6 mo. | 4/5 | 1/5 | — | — | — | — |
| 12 " | 2/3‡ | 0/3 | — | 0/2 | 0/2 | — |

*Horse-serum dilution.
†8 of 10 pigs with necrosis.
‡Hemorrhage within 3 hours followed by necrosis.

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[†] The advantages of the neck as a site of injection are: the distance from the site of skin-tests and the ample space for the lesion caused by the adjuvants. The pigs injected into the neck became slightly more sensitive than those injected into the thigh.

TABLE II.
Cutaneous Reactions One Year After Sensitization.

| After intracutaneous inj., hrs | Guinea pigs sensitized | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | with horse serum and adjuvants | | | | | | with horse serum alone | | | |
| | No. 1 | | No. 2 | | No. 3 | | No. 4 | | No. 5 | |
| | 1:1* | 1:10 | 1:1 | 1:10 | 1:1 | 1:10 | 1:1 | 1:10 | 1:1 | 1:10 |
| 3 | 35x35x3†<br>H 8x4 | 35x35x3 | 40x40x2<br>H 15x8 | 30x15x2<br>H 8x4 | 30x15x4<br>H 10x5 | 30x30x3 | 0 | 0 | 0 | 0 |
| 24 | 50x45x3<br>H 8x4 | 40x40x4<br>H 6x4 | 50x50x10<br>H 8x5 | 20x20x1 | 75x50x5 | C | 15x15x1<br>I.d. | 0 | 25x25x1<br>I.d. | 0 |
| 48 | 75x55x4<br>N 5x4 | C | 80x60x10<br>N 9x9 | C | 65x60x4 | C | 0 | 0 | 0 | 0 |
| 72 | 80x60x4<br>N 5x4 | C | 50x45x5<br>N 5x5 | scaling<br>4x4 | scaling<br>5x5 | 0<br>C | 0 | 0 | 0 | 0 |

*Serum dilution.
†Diameter of redness and swelling in mm.
H—hemorrhage.
N—Necrosis.
C—Confluent.
I.d.—Ill defined.

tion, whereas it was seen in the control group within 3 hours. With the former animals all the reactions reached their maximum in 48 hours, in the latter group in 24. However, in the second experiment all the reactions appeared within 3 hours in both groups of animals. (2) The disappearance of the reactions was strikingly different in the two groups. In the group with tubercle bacilli the reactions persisted for 72 hours or longer and in the control group disappeared within 48 hours. (3) There was a sharp difference as to the presence or absence of necrosis. Necrosis occurred frequently at the site of injections of undiluted and occasionally diluted (1:10) horse serum in the group with the adjuvants but never in the group injected with horse serum alone. (Table I.) (4) Reactions to higher dilutions of horse serum occurred more frequently in the group sensitized with the aid of adjuvants. In this group 6 of 10 pigs reacted to a 1:100 dilution of horse serum whereas in the control group only 2 of 10 (test one week after sensitization).

The striking results of skin-tests made one year after sensitization are shown in Table II.

In the second experiment 28 days after the initial injection precipitin-titrations were made using 1 ml horse-serum dilutions and 0.1 ml guinea pig serum. The results are shown in Table III.

The repeated intracutaneous test-injections probably contributed to the sensitization and precipitin-formation.

The mechanism of the effect of the adjuvants used is a complex one. Killed tubercle bacilli probably play the same rôle as living ones, though their rôle has not been elucidated; paraffin oil enhances the cellular reaction caused by tubercle bacilli and protects the bacteria from destruction and sustains sensitization and antibody-formation.[9] Aquaphor may have two effects. It may retard the possible separation of the horse serum, thus delaying destruction and elimination. It

TABLE III.

Precipitin-titers 28 Days After the Injection of

| | Horse serum and adjuvants | | | | Horse serum alone | | | |
|---|---|---|---|---|---|---|---|---|
| Guinea pig | 1:10 | 1:100 | 1:1000 | 1:5000 | 1:10 | 1:100 | 1:1000 | 1:5000 |
| 1 | +++ | + | Trace | Faint trace | Trace | + | 0 | 0 |
| 2 | +++ | ++ | + | 0 | + | Trace | 0 | 0 |
| 3 | +++ | ++ | Trace | 0 | + | Trace | 0 | 0 |
| 4 | +++ | ++ | + | 0 | + | Trace | 0 | 0 |
| 5 | +++ | ++ | + | 0 | Trace | Trace | 0 | 0 |
| 6 | — | — | — | — | 0 | 0 | 0 | 0 |

+++ Precipitate on the bottom of the tube.
— Not done.
0 Negative.

may be recalled that Ramon[10] has shown that the addition of lanolin to diphtheric toxin promotes antitoxin production; there are no data in Ramon's papers as to the duration of antitoxin-formation after the injection of toxin combined with lanolin.

*Conclusions.* When guinea pigs receive an injection of horse serum combined with a lanolin-like substance and killed tubercle bacilli suspended in oil, sensitization to horse serum is similar to that seen in guinea pigs injected with living tubercle bacilli and horse serum insofar as the reactions to the intracutaneous injection of horse serum lasts longer than 48 hours and may be necrotic. The duration of intense sensitization is remarkably long. Precipitin-titers are higher in guinea pigs immunized with the aid of adjuvants. Under the conditions of these experiments, the use of the lanolin-like substance seems to be essential.

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### 13626

## Concentration of Dilute Solutions of Virus of Mouse Encephalomyelitis by Pervaporation and Ultracentrifugation.*

Joseph L. Melnick.† (Introduced by Grover F. Powers.)

*From the Section of Preventive Medicine and the Department of Pediatrics, Yale University School of Medicine.*

The finding of the virus of poliomyelitis in sewage emphasizes the importance of methods for its detection in dilute, aqueous solutions.[1, 2] A satisfactory method should be able to concentrate the virus from dilute, non-infectious solutions into small infectious volumes, free from bacteria and chemical and bacterial toxins. To attain this end the experiments to be described represent an attempt to exploit the principles of pervaporation and ultracentrifugation.

Mouse encephalomyelitis virus (Theiler's mouse poliomyelitis virus) was chosen because its properties with respect to filtration, thermal inactivation, and stability to ether, and its capacity of producing an infectious myelitis in mice indicate a close similarity to

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[10] Ramon, G., Lemetayer, E., and Richou, R., *Rev. d'Immunol.*, 1937, **3**, 202.

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

† National Research Council Fellow in the Medical Sciences, 1941-42.

[1] Paul, J. R., Trask, J. D., and Gard, S., *J. Exp. Med.*, 1940, **71**, 765.

[2] Gard, S., *J. Exp. Med.*, 1940, **71**, 779; Trask, J. D., and Paul, J. R., *J. Exp. Med.*, 1942, **75**, 1.

human poliomyelitis virus.[3, 4] Moreover Olitsky and Schlesinger[5] have recently shown that in mice there are no essential histological differences between the neural lesions produced by Theiler's virus and those caused by the Lansing strain of poliomyelitis.

*Methods.* The virus used in these studies was the FA strain (which was kindly supplied to us by Dr. Max Theiler of the International Health Division of the Rockefeller Foundation). A 10% pooled brain extract, kept at –64°C in a solid $CO_2$ refrigerator, served as the source of the virus. For each experiment an aliquot of this extract was thawed, and a 4000 cc dilute, non-infectious solution of the virus ($10^7$-fold dilution of brain) made with distilled water. In all cases the volume of inoculum used was 0.03 cc; it was injected intracerebrally into Swiss mice (28-32 days old).[‡] The mice were observed for 20 days, and the time noted for the onset of encephalitic or myelitic symptoms. In the calculation of the average incubation time the survivors were considered to have remained well only 20 days.

Control titrations of each lot of virus were made to account for any loss in viral activity because of the time necessary for the concentration procedures. These control solutions were kept at 8 to 10°C until the concentrates were prepared. Both controls and concentrates were then injected within the same hour into groups of mice.

Criteria for the detection of the virus consisted of (a) the development (after a variable incubation period of at least several days) of weakness, ataxia, and, if death did not ensue, of paralysis of extremities and neck; and (b) the failure of brains of animals killed while sick to exhibit any bacterial growth when cultured on blood agar plates. Occasionally intracerebral passage of brain suspensions from sick animals was made, and histological sections of the brains and cords of sick mice were examined for lesions.

*Concentration by Pervaporation.* The passage of water through cellophane tubing placed before a fan (pervaporation) has been used for the concentration of protein from serum.[6] In the present experiments 4 cellophane tubes, 4.2 cm in diameter and 100 cm in

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[3] Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 49.

[4] Olitsky, P. K., *J. Exp. Med.*, 1940, **72**, 113.

[5] Olitsky, P. K., and Schlesinger, R. W., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 79.

[‡] All mice used were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine.

[6] Hartman, F. W., *J. Am. Med. Assn.*, 1940, **115**, 1989.

length, were filled with 4000 cc samples of the $10^7$-fold dilutions of the virus-infected brains. These were placed before 2 electric fans either in an air-conditioned room maintained at 23°C and 45% humidity, or in the laboratory at 18-23°C and about 30% humidity. Because of the rapid evaporation, the temperatures of the virus solutions were about 8 to 9° below that of the room temperature.

A typical experiment presented in Table II, indicates that the virus may be concentrated without inactivation by this method. Etherization (8%) appears to stabilize the virus during the time necessary for the concentration to take place, and the ether does not interfere with the passage of water through the membrane. In six experiments it has been possible to concentrate 4000 cc samples of inactive solutions into small active volumes (40 to 80 cc) in from 2 to 3 days.

*Concentration by Ultracentrifugation.* By means of the air-driven ultracentrifuge, it is possible to sediment the virus from extracts of infected brains (Table I). Gard and Pedersen[7] have utilized this technic in their isolation of the FA virus. The procedure adopted in the present work is illustrated in the following experiment.

October 13, 1941: 40 cc of a 10% brain suspension in 0.05 M sodium phosphate buffer at pH 8.0[§] were centrifuged in an angle centrifuge at 3000 rpm for 15 minutes. The opalescent supernatant fluid was then spun in a Beams ultracentrifuge at 45,000 rpm (142,000 times gravity) for 60 minutes. The 10° angle rotor of Masket[8] was used. After the run the water-clear supernatant fluid was collected, and the surface of the pellets and the sides of the tubes were washed 3 times with distilled water. The pellets were then extracted with 40 cc of pH 8.0 buffer, and the aggregated material was subsequently removed in the laboratory centrifuge. The data in Table I indicate that this pellet extract was equal in infectivity to the original 10% brain extract. Thus practically all of the virus must have been sedimented in the ultracentrifuge. The residual infectivity found in the 40 cc of the supernatant fluid reveals that only about 1% of the original activity failed to be sedimented.

Inasmuch as the above experiments show that the FA virus in brain extracts is large enough to be sedimented, ultracentrifugation was employed as a step in the method for concentrating dilute solu-

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[7] Gard, S., and Pedersen, K. O., *Science*, 1941, **94**, 493.

[§] A buffer at pH 8.0 was used in view of the fact that Theiler and Gard[3] found the FA virus to be most stable at this hydrogen ion concentration.

[8] Masket, V., *Rev. Sci. Inst.*, 1941, **12**, 429.

TABLE I.
Ultracentrifugation of Mouse Encephalomyelitis Virus.

| | Dilution of test sol. | Morbidity ratio* | Avg incubation time (days)† |
|---|---|---|---|
| Original extract (40 cc) | $10^0$ | 6/6 | 2.3 |
| | $10^1$ | 15/15 | 4.8 |
| | $10^2$ | 8/8 | 6.5 |
| | $10^3$ | 8/8 | 7.3 |
| | $10^4$ | 7/8 | 11.0 |
| Pellet " " | $10^1$ | 7/7 | 4.6 |
| | $10^3$ | 13/13 | 7.5 |
| Supernatant fluid " | $10^0$ | 8/8 | 5.5 |
| | $10^2$ | 9/13 | 14.1 |

*Denominator indicates number of mice inoculated intracerebrally; numerator indicates number of mice which developed symptoms compatible with encephalomyelitis.

†Incubation period to the onset of symptoms.

tions of the virus. After concentration by pervaporation of 4000 cc samples of $10^7$-fold dilutions of infected brains to from 40 to 80 cc, the resulting solutions were spun at 36,000 rpm for 100 minutes. No visible pellets were present in the lusteroid centrifuge tubes. The supernatant fluid was poured off; and the walls of the tubes were washed thoroughly (rubber-tipped glass rod) with a total volume of 1.0 cc or less of 0.05 M sodium phosphate buffer at pH 8.0. If the pervaporation had been allowed to take place in the presence of ether, the latter was removed *in vacuo* before ultracentrifugation.

The results of 4 experiments, one of which is cited in Table II, indicate that the FA virus even in dilute solutions may be sedimented to yield an active, although invisible, lining on the bottom surface of the ultracentrifuge tubes. The active material in 4000 cc samples of $10^7$ brain dilutions has been concentrated into fractions less than 1 cc in volume.

TABLE II.
Concentration of Mouse Encephalitis Virus by Pervaporation and Ultracentrifugation.

December 6, 1941. 4000 cc of a solution representing a $10^7$ fold dilution of infected brains were concentrated by pervaporation to 50 cc. The pervaporation concentrate was spun at 36,000 R.P.M. (92,000 times gravity) for 100 minutes, and the pellet extracted with 0.4 cc of 0.05 M phosphate buffer, pH 8.0.

| | Morbidity ratio | Avg incubation time |
|---|---|---|
| Control $10^3$ fold dilution of brain | 7/7 | 8.1 |
| " $10^5$ " " " " | 8/8 | 11.3 |
| " $10^7$ " " " " | 0/7 | |
| Pervaporation concentrate | 7/8 | 9.9 |
| Ultracentrifugal supernatant fluid | 2/9 | 19.2 |
| " pellet extract | 8/9 | 8.5 |

*Summary.* Mouse brains infected with the FA strain of Theiler's mouse encephalomyelitis virus have been diluted ten million times so that the solutions were no longer infective. By means of pervaporation it has been possible to concentrate 4000 cc samples of such inactive solutions into volumes of 40 to 80 cc. The virus present in the pervaporated solutions has been further concentrated by sedimentation in the ultracentrifuge into active fractions less than 1 cc in volume.

### 13627

## Identification of Amines in Anaerobic Kidney Extracts.

VICTOR A. DRILL. (Introduced by W. W. Swingle.)

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In recent studies on the formation of pressor amines by the kidney, Holz and Heise[1, 2] found that under anaerobic conditions, renal extracts could transform l-tyrosine and l-dihydroxyphenylalanine (l-dopa) into their corresponding amines. Bing and Zucker[3] reported that the decarboxylating enzymes in the kidney are specific for certain amino acids and that species differences exist. It was also found that the perfusion of kidneys with l-dopa, under conditions of oxygen lack, would result in the formation of a pressor substance, presumably hydroxytyramine.[4] Victor, Steiner and Weeks[5] prepared an anaerobic extract of kidneys that had marked pressor activity. Schroeder and Adams[6] found that tyrosinase would reduce the blood pressure of hypertensive dogs and rats, and would also abolish the pressor activity of Victor's extract, suggesting that the pressor activity of extract was due to amines. In view of this work it is of interest to determine the presence of amines in the extract of Victor, Steiner and Weeks, and to identify the amines if possible.

*Methods.* Extracts of ground hog kidneys were prepared ac-

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[1] Holz, P., *Klin. Woch.*, 1937, **16**, 1561.

[2] Holz, P., and Heise, R., *Arch. Exp. Path. und Pharm.*, 1939, **191**, 87.

[3] Bing, R. J., and Zucker, M. B., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 343.

[4] Bing, R. J., *Am. J. Physiol.*, 1941, **132**, 497.

[5] Victor, J., Steiner, A., and Weeks, D. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 767.

[6] Schroeder, H. A., and Adams, M. H., *J. Exp. Med.*, 1941, **73**, 531.

cording to the method of Victor, Steiner and Weeks,[5] being incubated under nitrogen at 37 C for 48 hours. Five kilograms of kidney were used in the preparation of each extract, but only 1 liter of Krebs bicarbonate buffer per kilogram of kidney was used in order to keep the final volume low. The extract was then treated according to the method of Barger and Walpole,[7] as follows: After incubation the extract was heated to 100°C with HCl to coagulate the proteins. The material was filtered by gravity and the filtrate evaporated to a thick syrup, which was then extracted with acetone, in which the hydrochlorides of the amines are soluble. After filtration the acetone was evaporated and the remaining liquid mixed with chloroform, any insoluble material being discarded. The chloroform solution was then extracted with dilute HCl. Four cc of the HCl extract (equivalent to 10 g of original kidney) were neutralized and injected intravenously into anesthetized dogs. The blood pressure rose an average of 100 mm of Hg in 3 minutes.

The acid solution of the bases was then made alkaline with NaOH, extracted 5 times with ether, and the ether solution dried with sodium sulphate. A solution of oxalic acid in dry ether was then added to the ether extract of the bases and a precipitate was obtained. The oxalate which precipitated was removed and crystallized from alcohol-acetone. After recrystallization the oxalate melted, with some decomposition, at 165°C. It gave a negative xanthroproteic and Millon test. Barger and Walpole[7] found the oxalate of isoamylamine to melt at 166°C. The free amine was obtained and converted to the hydrobromide which melted at 226°C. The amine was, therefore, isoamylamine.

After the ether extraction above, the solution was made N/2 to NaOH and extracted 4 times with amyl alcohol. The amyl alcohol extracts were treated according to Barger and Walpole's procedure[7] in an effort to obtain phenylethylamine. The final extract contained pressor activity, probably phenylethylamine, which was not positively identified. The solution gave a negative Millon test, showing the absence of p-hydroxyphenylethylamine.

The N/2 NaOH solution was then neutralized and made slightly alkaline with sodium carbonate, and again extracted with amyl alcohol. The amyl alcohol was removed under vacuum and after recrystallization as the hydrochloride the material melted at 268°C. Johnson and Daschavsky[8] give 269.5-270°C as the melting point of their tyramine hydrochloride and Barger[9] reports 268°C. The solution gave a strong Millon reaction. The base was benzoylated (Shotten-Bauman process) and a benzoyl derivative obtained which melted at 168° C after recrystallization from alcohol-water. This is the melting point of dibenzoyl-p-hydroxyphenylethylamine and identifies the pressor material as tyramine (p-hydroxyphenylethylamine).

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[7] Barger, G., and Walpole, G. S., *J. Physiol.*, 1908, **35**, 343.

[8] Johnson, T. R., and Daschavsky, P. G., *J. Biol. Chem.*, 1925, **62**, 725.

[9] *Cf.* reference 8.

*Discussion.* Victor, Steiner, and Weeks[5] pointed out that the pressor material in their extract would pass through a collodion membrane, and would withstand 20 minutes of boiling. They also raised the question whether or not the active substance was a pressor amine. The analyses reported in this paper show the presence of isoamylamine and tyramine, with the probable presence of some phenylethylamine in the extracts. Thus anaerobic kidney extracts contain pressor amines whereas under aerobic conditions no pressor amines are produced.[5] Any amines present under aerobic conditions are probably oxidized by amino-oxidases.[10] This suggests that amines may be formed in the kidneys of dogs rendered hypertensive by the Goldblatt clamp. It has been observed that the release of a renal ischemia after ½ to 24 hours will produce a rise in blood pressure,[11-13] and Bing and Zucker[14] have produced acute renal hypertension by injecting l-dopa into the ischemic kidney of the cat. It is also of interest that tyrosinase will inactivate angiotonin under certain conditions.[6]

*Summary.* The pressor effects of anaerobic kidney extracts prepared according to the method of Victor, Steiner, and Weeks are probably due to pressor amines. Tyramine and isoamylamine have been identified in the extracts and the probable presence of phenylethylamine has been shown.

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[10] Holz, P., Credner, K., and Walter, H., *Z. physiol. Chem.*, 1939, **262**, 111.

[11] Taquini, A. C., *Am. Heart J.*, 1940, **19**, 513.

[12] Friedberg, L., Landowne, M., and Rodbard, S., *Am. J. Physiol.*, 1940, **129**, 358.

[13] Collins, D. A., and Hamilton, A. S., *Am. J. Physiol.*, 1940, **130**, 784.

[14] Bing, R. J., and Zucker, M. B., *J. Exp. Med.*, 1941, **74**, 235.

### 13628

# Electroencephalographic Changes Induced by Intravenous Sodium Amytal.*

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Observations were made on the human electroencephalogram prior to and following the intravenous administration of sodium amytal. For this study, young dementia praecox patients were chosen as the experimental subjects.

*Method.* Thirty-six electroencephalograms were made on 25 individuals. The minimum recording time for each record was 30 minutes. Ten tracings were made with a single-channel, push-pull amplifier of our own construction. The characteristics of this instrument have previously been described.[1] For the remainder of the records, a three channel electroencephalograph was used. This instrument, basically similar to the one-channel apparatus, was obtained from Mr. Albert M. Grass of Boston. These matched amplifiers and recording systems show an essentially flat frequency-response of from 1 to 48 cycles per second for a sinusoidal signal. The over-all discharge time of these amplifier systems, for a D.C. pulse, is approximately 0.3 of a second. The time-axis was maintained at 30 mm per second by a synchronous motor. In all experiments, the recorders were electrically over-damped, so as to reduce the high-frequency oscillations of extra-cortical origin. This over-damping is evident from the character of the pulse-calibrating signal. Records taken with the one-channel and with the three-channel instrument do not differ in any essential detail.

Each test was performed with the subject resting on a couch placed inside a copper-screened enclosure. Electrodes consisted of flattened solder pellets measuring approximately 4 mm in diameter. The electrodes were placed on the scalp along symmetrical parasagittal lines. Both bi-polar and uni-polar recordings were made. The different methods of derivation gave rise to essentially similar findings.

In 3 of the experiments, continuous records were made while sodium amytal was slowly injected. In the other 33 experiments, electrographic recording was discontinued during the actual time of

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* This work is in part aided by the Supreme Council, 33° Scottish Rite, Masons of the Northern Jurisdiction, U. S. A.

[1] Cohn, R., and Katzenelbogen, S., *U. S. Naval Med. Bull.*, 1939, **37**, 596.

injection. The average dose of sodium amytal was 0.3 g; the extremes were 0.1 and 0.5 g.

*Results.* The preinjection, or control, wave-patterns ranged from a dominant, well-modulated, 8-12 wave per second type, to a pattern distinguished by random-frequency, low-amplitude oscillations. Irrespective of the type of presodium amytal record, the electrophysiologic response to the intravenous drug was grossly similar in 32 of the 36 records. The characteristic change in the wave-pattern evoked by sodium amytal is the appearance of sequences of higher-frequency waves, whose duration ranges from 40 to 50 milli-seconds (20-25 waves per second). In Fig. 1 the dominant alpha-pattern in

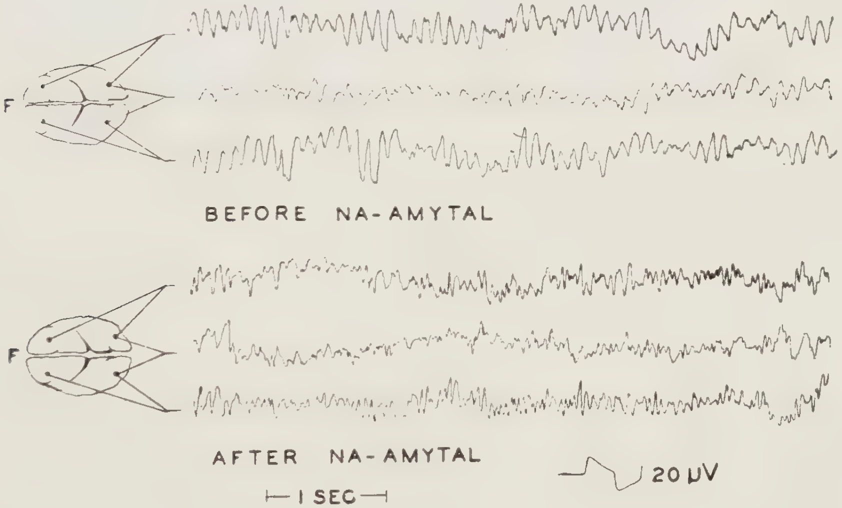

FIG. 1.

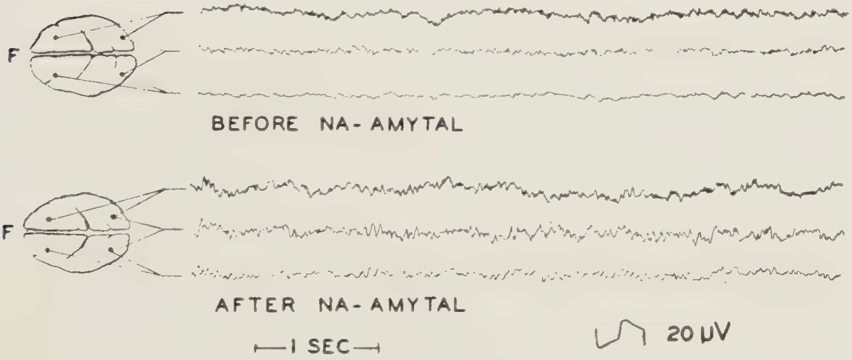

FIG. 2.

the simultaneous leads is almost completely replaced by the characteristic higher frequency pattern.

Fig. 2 shows the development of a rhythmic wave-pattern, following the intravenous injection of sodium amytal, in a previously low-voltage, random type of electroencephalogram. In this illustration, despite the lower amplitude as compared to the first figure, the frequency band is again closely regulated between 20 and 25 waves per second.

In the continuous records, that is, those made while actually administering sodium amytal, it was observed that short but definite sequences of approximately 20-wave-per-second discharges occurred within two minutes after the end of the injection. In all the records showing the characteristic sodium amytal response the maximum of the 20-wave-per-second activity occurred from 5 to 15 minutes after the drug was introduced. The wave-pattern returned to its "control" type in from 30 minutes to one hour. This recovery time appears to be a function of two main variables; the dosage, and the susceptibility of the individual to the drug.

In no instance did the slow intravenous administration of sodium amytal give rise to a more prominent alpha-pattern. However, in a few experiments the 20-wave-per-second pattern disappeared, or became inconspicuous, upon the appearance of the low-frequency pattern associated with sleep.

*Discussion.* A characteristic change in the electroencephalogram has been observed in 32 of 36 records after the intravenous administration of sodium amytal. This characteristic change is recognized by the appearance of a rhythmic wave-pattern whose frequency closely approximates 20 waves per second. This relatively high frequency pattern manifests itself irrespective of the type of the "spontaneous" electroencephalogram.

Several workers,[2–5] studying the effect of various barbiturate derivatives on the corticogram of animals, observed the appearance of higher frequency wave-forms in stages of light anesthesia. The frequencies noted by these workers ranged from around 15 to 30 waves per second. On the other hand, high frequency waves have not been reported in the brain potential patterns of human beings sub-

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[2] Derbyshire, A. J., Rempel, B., Forbes, A., and Lambert, E. F., *Am. J. Physiol.*, 1936, **116**, 577.

[3] Klaue, R., *J. f. Psychol. und Neurol.*, 1937, **47**, 510.

[4] Drohocki, Z., and Drohocki, J., *Compt. Rend. de la Soc. de Biol.*, 1939, **132**, 494.

[5] Beecher, H. K., and McDonough, F. K., *J. Neurophysiol.*, 1939, **2**, 289.

jected to intravenous sodium barbiturates.[6-9] In fact, it is stated or implied that there is really an enhancement of the basic alpha pattern following the use of sedatives. This phenomenon, as previously noted, has not been observed in our experiments.

The characteristic wave pattern of approximately 20 waves per second which results from the intravenous injection of sodium amytal is apparently not specific for the barbiturate derivatives. Similar rhythmic, high frequency, electric activity has been observed in certain phases of cyclopropane anesthesia,[10] and in early sleep.[11, 12]

*Conclusion.* There is a definite and characteristic change in the human electroencephalogram following the slow intravenous administration of sodium amytal. The change is marked by the early appearance of a rhythmic oscillation, with a frequency of approximately 20 waves per second, in place of the individual's "spontaneous", or control, pattern. This action is not specific for the barbiturate derivatives. It is a general concomitant of early "depressed" cortical activity.

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## 13629

## Cine-Photomicrographic Apparatus With Constant Temperature Chamber for Tissue Culture Studies.*

MACHTELD E. SANO AND EDWIN S. GAULT. (Introduced by L. W. Smith.) (With the technical assistance of Mr. James Davies.)

*From the Department of Pathology, Temple University School of Medicine, Philadelphia.*

Sano and Smith[1] reported a simplified technic for tissue culture,

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[6] Lennox, W. G., Gibbs, F. A., and Gibbs, E., *Arch. Neurol. Psychiat.*, 1936, **36**, 1236.

[7] Gibbs, F. A., Gibbs, E. L., and Lennox, W. G., *Arch. Int. Med.*, 1937, **60**, 154.

[8] Lemere, Frederick, *J. Neurophysiol.*, 1939, **1**, 590.

[9] Penfield, W., and Erickson, T. C., Chapter 14 by H. H. Jasper, *Epilepsy and Cerebral Localization*, 1941, Charles C. Thomas, Springfield, Illinois.

[10] Rubin, M., and Freeman, H., *J. Neurophysiol.*, 1940, **3**, 33.

[11] Davis, H., Davis, P. A., Loomis, A. L., Harvey, E. N., and Hobart, G. A., *J. Neurophysiol.*, 1938, **1**, 24.

[12] Blake, H., Gerard, R. W., and Kleitman, N., *J. Neurophysiol.*, 1939, **2**, 48.

* Aided by a grant from the Clinical Research Foundation of Philadelphia.

We are indebted to Dr. Lawrence W. Smith and Dr. George C. Henny for their valuable suggestions and help.

[1] Sano, Machteld E., and Smith, Lawrence W., PROC. SOC. EXP. BIOL. AND MED., 1930, **28**, 282.

utilizing a glass (or metal) ring with 2 cover slips as the chamber, this is in turn placed within an ordinary Petri dish for convenience in handling and maintaining sterility. It possessed the advantages of easy accessibility to, and a flat surface for microscopic study of, the culture. The obvious desirability of being able to obtain permanent cinematographic records prompted the authors to devise a compact

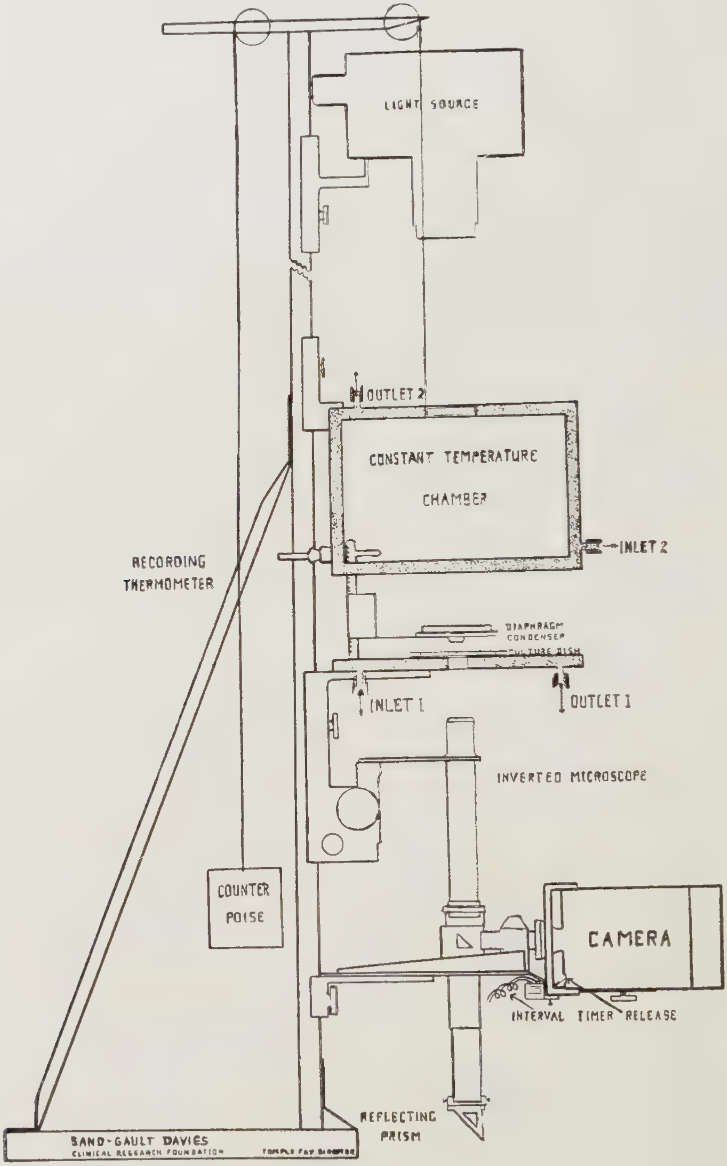

Fig. 1.

and simple apparatus in which constant temperature of any desired degree could be maintained, and with which filming of the culture could be carried out at will. Because of the apparent widespread interest in the apparatus, as expressed by many fellow investigators in this field, we felt it might extend its field of usefulness if we published a brief memorandum regarding its construction. A diagrammatic sketch, Fig. 1, is appended to make clear the mechanism. It should be mentioned that this is an original experimental model, and that certain obvious refinements could be incorporated in any subsequent unit.

Actually the apparatus was built around our special tissue culture dish, to permit the culture to remain in its normal upright position. This required the inversion of the microscope in order to obtain high power photomicrographs, as otherwise the depth of the culture chamber interfered with any but extremely low magnification projection. Partly to decrease the length of the optical system and partly because a side arm viewing prism was not available, the camera (a Bell-Howell 16 mm cartridge type) was interposed horizontally by the intervention of a prism in the projection system (see diagram). The light source was a standard Bausch and Lomb ribbon filament lamp of the projection type. So far as the optical system is concerned there is nothing unusual about it other than its inverted position.

The constant temperature chamber (or incubator as it might be termed) is the important feature of the apparatus. This consists of a hollow stage, and a double walled, square, box-like cover which fits closely over the stage, measuring 0.8 cm in total thickness. The stage and the roof of the cover each have a window to permit illumination. The cover is suspended by a steel wire which is counterbalanced, thus making the chamber instantly accessible. Fluid is circulated through the hollow walls of the stage and cover at any desired temperature, and this temperature level can be maintained with no more than ±0.2° variation. The condenser rack and pinion system was relocated, so as to be wholly within the constant temperature chamber.

The temperature of the fluid is maintained constant by the use of some type of mechanical compressor. If only temperatures in the lower range are needed any ordinary electric refrigerator unit could be used. Actually the equipment which we have used is a small bedside unit made by the Therm-O-Rite Corporation of Buffalo, N. Y., for the clinical studies relating to hypothermy carried out under the direction of Dr. Temple Fay.[2] Using freone as the refrigerant and by inserting a heating unit in the same machine a temperature range of over 100°C can be obtained which will remain constant within very narrow limits.

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[2] Fay, Temple, *Proc. Inter-State Postgr. Med. Assn. N. Am.*, Oct. 14-18, Cleveland, Ohio, 1940.

A resistance thermometer with a self-recording mechanism (Micromax-Leeds and Northrup Company, Philadelphia) is introduced into the chamber so that any deviation from the constant temperature may be noted, and the cultural results interpreted accordingly.

An interval timer with an automatic release is attached to the camera which permits a wide range of interval-timed photography ranging from seconds to hours. The most practical of such apparatus we have found to be the extremely compact unit made by J. W. Robbins of Philadelphia. In this way the entire procedure is automatic with the exception of critical focusing. Particularly with rapidly growing cells such focusing must be carried out at frequent intervals to secure satisfactory photographic results. In our apparatus this can be done directly through the microscope eyepiece or with the intervention of a removable prism in the system which makes the focusing somewhat more convenient.

## 13630

## Susceptibility of Syrian Hamsters to Leptospirosis.

HARRY E. MORTON. (Introduced by Stuart Mudd.)

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Packchanian[1] studied the susceptibility of 32 species and subspecies of rodents to virulent strains of *Leptospira icterohemorrhagiæ* and concluded that certain species of American deer-mice, genus *Peromyscus,* are suitable as susceptible small laboratory animals for experimental studies of icterohemorrhagic spirochetosis and for the diagnosis of Weil's disease. He did not, however, include hamsters in his studies. Larson[2] reported that white mice *(Mus musculus)* are extremely sensitive to *L.icterohemorrhagiæ.* He emphasized that young animals be employed. The mortality rate approximates 100% in 3-week-old mice, but falls rapidly as age increases. At this time we wish to report on the susceptibility of Syrian hamsters to *L. canicola* and *L. icterohemorrhagiæ.*

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[1] Packchanian, A., *Pub. Health Rep.,* 1940, **55**, 1389.

[2] Larson, C. L., *Pub. Health Rep.,* 1941, **56**, 1546.

The Syrian hamsters were obtained originally from H. C. Schaefer, Research Department of Ralston Purina Co., St. Louis. The strain of *L. canicola* was obtained from Dr. Karl F. Meyer, George Williams Hooper Foundation, San Francisco. It was isolated from dog blood by culture methods. The strain of *L. icterohemorrhagiæ* was obtained from Dr. Ruth E. Miller, Woman's Medical College of Pennsylvania, Philadelphia. It was isolated from rats in Philadelphia. The culture medium was Schüffner's modification of Verwoort's medium, as described by Meyer, Stewart-Anderson, and Eddie.[3]

*L. icterohemorrhagiæ:* 0.5 ml of cultures which had been incubated approximately one week at room temperature, when injected subcutaneously into hamsters 3 to 5 weeks old, produced death in 5 to 8 days. Cultures made from the heart's blood 24 hours after the injection remained sterile, but those made 48 and 72 hours after the injection were positive for leptospira. Animals not over 5 weeks old should be used if it is desired to produce death of the animals with typical signs of icterus.

*L. canicola:* 0.5 ml of cultures which had been incubated approximately one week at room temperature, when injected subcutaneously into hamsters 3 to 5 weeks old failed to bring about death of the animals. Cultures made from the heart's blood 48, 72, and 96 hours after the injection were positive for leptospira whereas the cultures made 24 hours after the injection remained sterile.

Although our work has been limited to one strain each of the two species of *Leptospira*, it appears that Syrian hamsters may be useful as laboratory animals in working with *L. canicola* and *L. icterohemorrhagiæ*.

Taylor[4, 5] reported that Syrian hamsters, in addition to ferrets, gave an immune response to influenzal virus in human throat washings, and since our first use of these animals in April, 1941, other reports have appeared on their usefulness as laboratory animals. Broun, Muether, Mezera, and LeGier[6] found them highly susceptible to the virus of St. Louis encephalitis and that humoral antibodies were demonstrable in the blood sera if the animals survived 7 days or longer.[7] Eaton, Martin and Beck[8] reported the hamsters susceptible to the viruses of meningopneumonitis and lymphogranuloma venereum.

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[3] Meyer, K. F., Stewart-Anderson, B., and Eddie, B., *J. Am. Vet. Med. Assn.*, 1939, **95**, 710.

[4] Taylor, R. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 541.

[5] Taylor, R. M., and Parodi, A. S., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 105.

[6] Broun, G. O., Muether, R. O., Mezera, R. A., and LeGier, M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 601.

## 13631 P

## Blood Cultures from Pulmonary Artery and Aorta in Patient with Infected Patent *Ductus arteriosus.*

ARTHUR S. W. TOUROFF. (Introduced by G. Shwartzman.)

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Operation, in cases of subacute bacterial endarteritis superimposed on patent *ductus arteriosus*, necessitates direct exposure of the pulmonary artery and aorta.[1, 2, 3] This offers an opportunity to take blood cultures directly from the above structures.

In a recent case, 14 ml of blood were drawn simultaneously from both the pulmonary artery and the aorta. Agar-plate cultures of the blood taken from the pulmonary artery revealed innumerable colonies of *Streptococcus viridans*. The aortic specimen contained 51 colonies per ml of blood. These quantitative determinations indicate that the bacterial content of the blood *entering* the lungs differs from that of the blood *leaving* the lungs (and traversing only the left side of the heart.).

Heretofore the existence of a pulmonary protective barrier, which removes infective material from the circulating blood, has been demonstrated directly only in experimental animals.[4-9] Furthermore, in cases of subacute bacterial endarteritis superimposed on patent *ductus arteriosus*, it has not been shown as yet whether infective material derived from vegetative foci within the *ductus* and the adjacent portions of the pulmonary artery, reaches the peripheral circulation by passing through the pulmonary circuit or by entering the aorta through the *ductus,* or both.

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[7] Broun, G. O., LeGier, M., Mezera, R. A., and Muether, R. O., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 310.

[8] Eaton, M. D., Martin, W. P., and Beck, M. D., *J. Exp. Med.*, 1942, **75**, 21.

[1] Touroff, A. S. W., and Vesell, H., *J. A. M. A.*, 1940, **115**, 1270.

[2] Touroff, A. S. W., and Vesell, H., *J. Thoracic Surg.*, 1940, **10**, 59.

[3] Touroff, A. S. W., Vesell, H., and Chasnoff, J., *J. A. M. A.*, 1942, **118**, 890.

[4] Wyssokowitsch, W., *Z. f. Hyg.*, Leipzig, 1886, **1**, 3.

[5] Werigo, M., *Ann. l'Inst. Pasteur*, 1894, **8**, 1.

[6] Bull, C. G., *J. Exp. Med.*, 1915, **22**, 475.

[7] Aschoff, L., *Z. exp. Med.*, 1926, **50**, 52.

[8] Christeller, E., and Eisner, G., *Beitr. Z. Path. Anat. u. z. Allg. Path.*, 1929, **81**, 524.

[9] Seeman, G., and Theodorowitsch, W., *Ztschr. f. d. Ges. Exp. Med.*, 1929, **69**, 742.

The observations described herein demonstrate directly, that: (1) The lungs play an important rôle in removing infective material from the circulating blood of humans; and (2) in cases of subacute bacterial endarteritis superimposed on patent *ductus arteriosus,* infective material enters the peripheral circulation, at least in part, through the pulmonary circuit.

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## 13632

## Glucoside Type of Cerebroside in the Spleen in Gaucher's Disease.

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It has been generally assumed that the cerebroside found in the enlarged spleen characteristic of Gaucher's disease is the usual galactoside type of kerasin. The principal published evidence for this concept is the melting point of the phenylosazone prepared from the cerebroside hydrolysate.[1] However, Halliday, *et al.,*[2] proved by fermentation and by the properties of the phenylosazone that the kerasin-like cerebroside in one case of Gaucher's disease contained *d*-glucose rather than *d*-galactose. The spleen used by these investigators had been preserved in formalin for several weeks.

We wish to report the isolation of glucose-containing cerebroside from a fresh spleen which was removed surgically from a four-year-old girl with Gaucher's disease. The clinical diagnosis was confirmed by pathological examination of the spleen which weighed 530 g.

The cerebroside was isolated by the method of Kaye[3] (maceration of the spleen with plaster of Paris, extraction of the powdered mass with three parts of boiling 95% ethyl alcohol, filtration, and crystallization of the cerebroside by cooling the filtrate below 0°C). The yield of crude cerebroside from a single extraction was equivalent to 1.17% of the moist spleen. The cerebroside was purified by extraction with ether for four hours, and by repeated recrystallization from hot methyl alcohol. The purified cerebroside was white and had a melting point of 175°C.

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[1] Lieb, H., *Z. physiol. Chem.,* 1924, **140,** 305; Lieb, H., and Mladenovic, M., *Z. physiol. Chem.,* 1929, **181,** 208.

[2] Halliday, N., Deuel, H. J., Jr., Tragerman, L., and Ward, W. E., *J. Biol. Chem.,* 1940, **132,** 171.

[3] Kaye, I. A., *J. Lab. Clin. Med.,* 1940, **25,** 1117.

The method of Rosenheim[4] was used for hydrolysis of the cerebroside. A solution of 1 g in 50 ml of hot methyl alcohol containing 5 ml of concentrated sulfuric acid was refluxed 6 hours on a water bath. After cooling below 0°C for fifteen hours, the methyl ester of lignoceric acid was removed by filtration. The filtrate was diluted with one-half volume of water containing sufficient sulfuric acid to produce a 10% solution, and it was then hydrolyzed 10 hours on a boiling water bath. After neutralization with silicate-free potassium hydroxide to bromthymol blue, a tarry mass of sphingosine sulfate separated and was removed by filtration. The hydrolysate was diluted to a volume of 100 ml, and aliquots were used for identification of the carbohydrates. All procedures and analyses were conducted in duplicate.

The reducing equivalents of the carbohydrate were determined by the Folin-Wu[5] and Sumner[6] methods, a 6 to 100 dilution of the neutralized hydrolysate being used for the former, and a 1 to 2 dilution for the latter. The respective equivalents were found to be 1.50 and 1.53 mg of glucose per ml of hydrolysate. The Sumner/Folin-Wu ratio (0.98) was within experimental error of the characteristic ratio for *d*-glucose (1.00), and it was far removed from the ratio for *d*-galactose (1.24).[7, 8] On the basis of an estimated 70% recovery in the hydrolysate,[2] the cerebroside contained 21.9% of *d*-glucose. The reducing sugar in the hydrolysate was completely removed by short-period fermentation with fresh Fleischmann's yeast which had been washed 5 times with distilled water. The optical rotation of the neutralized hydrolysate was determined in a Schmidt and Haensch polariscope with an electric sodium lamp. The calculated specific rotation was +47.6°, as compared with +53.4° for a glucose solution subjected to similar hydrolytic treatment. The discrepancy between the two values was equivalent to a difference in observed rotations of only 0.04°. The presence of *d*-galactose was excluded by these results.

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[4] Rosenheim, O., *Biochem. J.*, 1913, **7**, 604.

[5] Folin, O., *J. Biol. Chem.*, 1920, **41**, 367.

[6] Sumner, J. B., *J. Biol. Chem.*, 1925, **65**, 383.

[7] Everett, M. R., Edwards, B., and Sheppard, F., *J. Biol. Chem.*, 1934, **104**, 11.

[8] Everett, M. R., and Sheppard, F., *J. Am. Chem. Soc.*, 1938, **60**, 1792.

*Conclusions.* The isolated cerebroside was not the customary kerasin; it contained *d*-glucose in place of the usual *d*-galactose component, as proven by fermentation, optical rotation and reducing equivalents. Halliday, *et al.*, suggested that the cerebroside isolated by them might represent an anomaly of carbohydrate metabolism. Our results, and those recently reported by Klenk and Schumann,[9] indicate that synthesis of a glucoside type of splenic kerasin is a frequent occurrence in Gaucher's disease.

### 13633

## Electrical Properties of Tissues in Shock Therapy.

Franklin Offner. (Introduced by R. W. Gerard.)
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A lack of knowledge of the exact treatment current has hindered the standardization of electrical shock therapy. This has been especially true because the treatment current does not appear to follow "Ohm's law"—that is, the current is not equal to the treatment voltage divided by the measured (d-c) resistance between the electrodes, but usually is considerably higher. Also, the resistance varies in what has appeared to be an "unpredictable" manner. For these reasons, an *a priori* selection of the treatment current has appeared to be impossible.

It is, of course, the passage of the electric current which is responsible for the convulsive shocks, rather than the applied voltage of itself, so that dosage standardization must be on the basis of the former. The voltage required to obtain a given current will depend upon many factors, such as the thickness of the skull, area of electrodes, condition of the skin, etc. Obviously a rational prescription of dosage requires some method of taking these factors into account. This was recognized even in the original work of Cerletti and Bini (1938), who employed a d-c resistance measurement as mentioned above. However, this bears no relationship to the effective resistance during treatment.

A study of the electrical properties of the tissues involved offers an explanation of the apparently anomalous behavior of the resistance. This has made possible the accurate preselection of the treatment current used in shock therapy.

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[9] Klenk, E., and Schumann, E., *Z. physiol. Chem.*, 1940, **267**, 128.

*Method.* Two electrodes were placed on the subject's head, using electrode jelly, as for shock treatment. The alternating current impedance between these was measured at frequencies of from 50 to 15,000 c.p.s. by means of an impedance bridge.* The very low current employed produced no stimulation. The alternating current impedance of tissue may be separated into two components: the resistance or energy dissipating portion; and the reactive, or energy storing portion. The latter results from the polarizability of living tissue.

Considerable information concerning the electrical behavior—and also at least to some extent the structure—of tissue may be obtained by plotting the "impedance locus" (Cole and Curtis[1]). In this, the reactance is plotted against the resistance for each frequency at which the impedance is measured. Proper analysis will then frequently allow the derivation of an "equivalent electrical network," in which the electrical properties of the tissues are represented by a combination of ordinary electrical components, such as resistors and condensers.

*Electrical Results.* A typical impedance locus as obtained in the above measurements is illustrated in Fig. 1. An analysis of the figure shows that these properties may be approximated by the network of Fig. 2, with $R_1$ 100 ohms, and $R_2$ 500 ohms. C is the element of the network introducing the reactance, and represents the membrane polarizability. It is a condenser of about 0.4 μfd capacity and 62° phase angle.

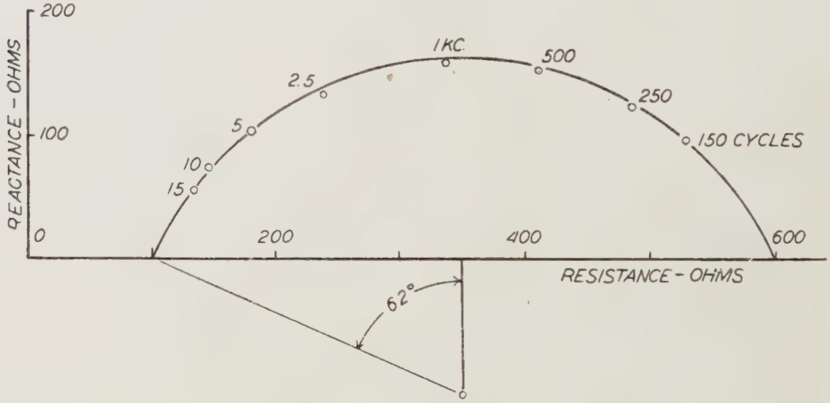

FIG. 1.
Impedance locus of electrodes.

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* Offner Electronics, Type 800.

[1] Cole, Kenneth, and Curtis, H. J., *Cold Spring Harbor Symposium,* 1936, **4**, 73.

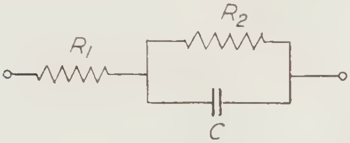

Fig. 2.
The equivalent circuit.

As direct currents cannot pass through a condenser, it is apparent that the d-c resistance of the network will be $R_1 + R_2$, or 600 ohms. This is the high resistance intercept of the impedance locus of Fig. 1—the "zero frequency extrapolation." Very high frequencies will, on the other hand, pass through C almost unimpeded, so that no current will flow through $R_2$. The effective network resistance will then be $R_1$, which is the low resistance intercept of the locus—the "infinite frequency extrapolation."

It has been shown (Cole and Curtis[2]) that electrically stimulated tissues lose almost completely their polarizability; that is, they become almost completely permeable. In the above network, this may be represented by $R_2$ falling to such a low value that it effectively short-circuits the condenser C. The measured resistance of the network at all frequencies will then be only slightly greater than $R_1$. Now the current used in shock therapy is of quite great strength, and it must stimulate the tissues carrying the bulk of the current. Thus during the passage of the treatment current, the resistance of $R_2$ will fall to a low value, and the effective resistance to the passage of the treatment current will be only slightly greater than $R_1$. It is apparent, however, that the *effective* resistance of the patient can be determined beforehand by use of a high frequency test current, rather than the direct current originally used. The following relationship may be stated: The measured electrode resistance before treatment, using a low intensity, high frequency testing current, should equal the resistance to the passage of the high intensity, low (60 c.p.s.) frequency treatment current.

The above relationship was used in setting the dosage in shock therapy. A 7000 cycle testing current is first passed through the electrodes on the patient. The voltage is adjusted until this testing current is a fixed fraction (about one ten-thousandth) of the desired treatment current; the treatment voltage to be given is simultaneously raised proportionately. When the shock is given, the treatment current should then bear this same relationship to the test current, and the test current meter may thus be calibrated directly in terms of the treatment current.

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[2] Cole, Kenneth, and Curtis, H. J., *J. Gen. Physiol.*, 1939, **22**, 37, 649.

Both because of its low intensity and because its frequency is remote from the pararesonance frequency of nerve cells (Monnier[3]), the test current cannot be felt by the patient.

The fact that the d-c resistance between the electrodes is almost meaningless became obvious during the impedance measurements; it was found that it could be changed at will by varying the technic used in electrode application. Such variations did not, however, appreciably affect the high frequency resistance. Nor has experience in administration of shocks indicated that they affect the value of the treatment current.

Convulsive shocks were administered to a number of patients with the instrument, as listed in Table I. In every instance the treatment current was very nearly equal to the value predicted by the test meter—always within 5%. The rather large internal stabilizing resistance of the instrument tends to obscure small differences between the patient's resistance to the testing and treatment currents. The results, however, confirm the conclusion that the effect of the tissue polarizability is largely eliminated by the flow of the treatment current.

The advantages of the use of this technic in the administration of electric convulsive shocks appears to be two-fold: it allows the value of the treatment current to be set at the value desired for the treatment; and the current values used by different workers can be compared. The voltage required to obtain a given current will vary with the exact form of electrode employed, as well as anatomical differences between patients. Thus it would be expected that considerable variation in voltage values would occur from subject to subject, as well as between different workers for the same subject. On the other hand, current values should be more nearly constant. It has, in fact,

TABLE I.

| Patient No. | Test current, ma. | Treat. current, ma. |
|---|---|---|
| 1 | 380 | 380 |
| 2 | 380 | 380 |
| 3 | 330 | 320 |
| 4 | 400 | 400 |
| 5 | 450 | 450 |
| 6 | 190 | 180 |
| 7 | 450 | 455 |
| 8 | 450 | 450 |
| 9 | 330 | 330 |

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[3] Monnier, A. M., *L'Excitation Electrique des Tissus,* Hermann et Cie, Paris, 1934.

been found that the large percentage of subjects will receive convulsions with currents of from 400 to 450 ma, applied for 0.2 or 0.3 seconds.

The writer wishes to thank Drs. E. Dombrowski and J. V. Edlin of the Chicago State Hospital for their coöperation in the test of this instrument, and Dr. R. W. Gerard for assistance in preparation of the manuscript.

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## 13634

# Influence of Hyperpyrexia on Ascorbic Acid Concentration in the Blood.

Stafford L. Osborne and Chester J. Farmer.

*From the Departments of Physical Therapy and Chemistry, Northwestern University Medical School, Chicago, Ill.*

It has been established[1, 2, 3] that the plasma ascorbic acid concentration during the course of an infectious fever is lowered, as a consequence, the therapeutic procedure of supplementing the diet of such patients with ascorbic acid is receiving increased attention.

This investigation was undertaken to ascertain whether an artificial fever produced by physical means also produced a lowering of the plasma ascorbic acid. When this work was initiated no such study had been reported; but in the meantime two somewhat similar investigations have been reported.[4, 5] In addition, Rhinehart's[6, 7] association of low plasma ascorbic acid levels with the etiology of rheumatic disease further augmented our interest, inasmuch as our patients had been afflicted with this disease.

*Method.* Seventeen patients suffering from chronic arthritis were subjected to hyperpyrexia. The body temperature was elevated and maintained at 104 to 104.6 F (rectal) for a period of 4 hours, following which it was permitted to return to normal. A combination of fever cabinet and inductotherm was used to elevate the patient's temperature. Treatments were given once weekly.

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[1] Farmer, C. J., *Quart. Bull. Northwestern Univ. Med. Sch.*, 1940, **14**, 220.

[2] Wolfer, J. A., *Surg. Clin. N. A.*, Chicago Number, Feb., 1940.

[3] Wolfer, J. A., *Surg. Gynec. Obst.*, 1939, **69**, 745.

[4] Daum, K., Boyd, K., and Paul, W. D., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 129.

[5] Zoak, J., and Sharpless, G. R., Proc. Soc. Exp. Biol. and Med., 1938, **39**, 233.

[6] Rinehart, J. F., *Ann. Int. Med.*, 1935, **9**, 586.

[7] Rinehart, J. F., *Ann. Int. Med.*, 1935, **9**, 671.

The blood plasma ascorbic acid concentration was determined by the micro method of Farmer and Abt.[8] In 2 of the patients with widely divergent initial plasma levels (0.36 and 0.60 mg % respectively) a supplement of 450 mg ascorbic acid (Cebione Merck*) was given daily for the purpose of observing differences in plasma level behavior under treatment with a more than adequate ingestion of vitamin C.

Seventy-seven observations were made on the 17 patients. The analytical determinations were made on each patient (a) just prior to treatment, (b) when the rectal temperature reached 104°F, (c) after a temperature of 104°F had been maintained for 4 hours, and (d) after the patient's temperature had returned to normal.

*Results.* Table I gives a summary of the average values and the result of the statistical analysis. Obviously, there are no significant changes in the blood plasma ascorbic acid cencentration during or following the course of the artificial fever.

TABLE I.
Summary of Blood Ascorbic Acid Determinations.

| | Elapsed time, hrs | Avg of 77 determinations on 17 chronic arthritis patients | σ ± | 2 γ diff. ± | Change ± |
|---|---|---|---|---|---|
| Control | 0 | .62 | .03 | | |
| Temp. reached 104° F. | 2 | .68 | .04 | .104 | .06 |
| Temp. at 104°F 4 hours | 6 | .66 | .03 | .106 | .04 |
| Temp. returned to normal | 8 | .64 | .03 | .088 | .02 |

*Discussion.* Investigators vary markedly in their reports as to what constitutes a normal blood plasma ascorbic acid level, either in health or disease. Abt and Farmer,[9] in their studies on children and young adults, report normal values for patients with adequate intake to range from 0.7 to 1.5 mg per hundred cubic centimeters. Blood plasma values below 0.7 mg they consider to be suboptimal. Furthermore, according to these investigators, active scurvy may occur in children with values up to 0.4 mg %. Abt and Farmer believe that while these values are critical during the period of growth, they may not hold the same significance in the adult. Wolff, Banning, and van Eekelen[10] state a blood level of 0.4 to 1.2 mg % to be adequate but not the optimum. Daum, Boyd and Paul[4] state that the normal ascorbic acid content of the blood ranges between 0.7 to 2.0 mg %. Ralli and Sherry[11] in their recent review agree that plasma levels above 0.7 mg % can be considered normal with a white cell platelet content of 25 to 38 mg %.

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[8] Farmer, C. J., and Abt, A. F., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 146.

* We are indebted to Merck and Company, Rahway, N.J., for the ascorbic acid (Cebione, Merck) used in this investigation.

[9] Abt, A. F., and Farmer, C. J., *The Vitamins,* Chapter 22, Am. Med. Assn. Press, Chicago, 1939.

While the average blood plasma ascorbic acid concentration for our group was 0.62 mg % before treatment, the range varied from a low of 0.28 to 0.72 mg %. Many of the values found in these patients are considered to be subnormal. Thirty-three of the 77 determinations before treatment showed a level below 0.5 mg %. This is of interest in the light of the work of Rinehart,[6, 7] who as the result of his study expressed the following opinion: "On the basis of experimental data previously reported, the concept was presented that rheumatic fever may be the result of the combined influence of vitamin C deficiency and infection. With respect to the rôle of infection, it may briefly be said that in the presence of adequate vitamin C nutrition, rheumatic type lesions have not been observed."

The claim has been made that fever increases the vitamin C requirement. The most worthy evidence in support of this claim has apparently been submitted by Zoak and Sharpless.[5] They subjected guinea pigs to a scorbutogenic diet and to artificial hyperpyrexia for from 2 to 6 hours daily. Early in the course of the experiment they found the vitamin C content of the tissues reduced by hyperpyrexia, but as the scorbutic state was approached, little difference was found. This was particularly true of the adrenals. The evidence obtained from human subjects is divergent. The conclusion of Zoak and Sharpless[5] that no significant change in the blood level of vitamin C occurs, was confirmed by our results. They believed the failure to observe a decrease was due to hemoconcentration; however, our studies[12] failed to show a hemoconcentration during hyperpyrexia when produced by the methods and management employed. On the contrary, Daum, Boyd and Paul[4] observed a decrease in the blood plasma level. Falke,[13] Daum, Boyd and Paul[4] and Zoak and Sharpless[5] studied the urinary excretion of vitamin C as well as the blood level. These authors with the exception of Zoak and Sharpless interpreted their data as showing that fever increases the need or utilization of vitamin C in man. According to Falke,[13] who induced a fever with "pyrifer", the need is increased by 100 mg per day. Zoak and

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[10] Wolff, L. K., Banning, C., and van Eekelen, M., *Quart. Bull. Health Organ. League of Nations,* 1936, **5**, 566.

[11] Ralli, E. P., and Sharry, S., *Med.,* 1941, **20**, 251.

[12] Osborne, S. L., *Arch. Physical Therapy,* 1941, **22**, 407.

[13] Falke von, *Klin. Wchnschr.,* 1939, **18**, 818.

Sharpless expressed the view that by following the blood level and urine excretion one cannot accurately determine if fever increases the utilization of vitamin C; tissue analysis as performed in the guinea pig studies would seem to yield more direct evidence. The reliability of data on the urinary excretion in regard to the utilization or need for vitamin C is questioned by Abt and Farmer[9] and Wolff, Banning and van Eekelen.[10] The blood plasma level would appear to be more reliable, since the level at which vitamin C appears in the urine is subject to considerable individual variation. Further, Hawley, Frazer, Button and Stephens[14] present evidence indicating that the reaction of the urine is concerned in determining the excretion of vitamin C. Finally, in man, considerable vitamin C is lost through the sweat according to the observations of Cornbleet, Klein and Pace.[15] This, however, is denied by Wright and MacLenathan.[16] Since we could not find a satisfactory method applicable to our patients for the determination of the loss in the sweat, we relied on the assumption that the blood plasma level is the best indicator for the body level of vitamin C. According to this view, a body temperature of from 104° to 104.6°F for 4 hours did not significantly or detectibly increase the rate of utilization of vitamin C.

*Conclusions.* A study of 77 observations made on 17 patients showed no significant changes in the ascorbic acid concentration as the result of elevating the body temperature to 104°F and maintaining it for 4 hours, using a combination of air conditioned cabinet and inductotherm.

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## 13635 P

## Destruction of Phenylalanine by Ultraviolet Radiant Energy.

L. EARLE ARNOW.

*From the Laboratory of Physiological Chemistry, University of Minnesota, Minneapolis.*

The following changes have been observed to occur when *dl*-phenylalanine is exposed to ultraviolet radiant energy:

1. Spiegel-Adolf[1] reported that solutions of phenylalanine ex-

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[14] Hawley, E. E., Frazer, J. P., Button, L. L., and Stephens, D. J., *J. Nut.*, 1936, **12**, 215.

[15] Cornbleet, T., Klein, R. J., and Pace, E. R., *Arch. Derm. Syph.*, 1936, **34**, 253.

[16] Wright, I. S., and MacLenathan, E., *J. Lab. and Clin. Med.*, 1939, **24**, 806.

[1] Spiegel-Adolf, M., *Biochem. J.*, 1937, **31**, 1303.

posed to ultraviolet rays become pigmented. Pigment formation was most marked in neutral solution, but acid and alkaline solutions also became red-brown after prolonged irradiation. During the present investigation, these findings were confirmed. Moreover, when dilute sulfuric acid or hydrochloric acid solutions of the amino acid were exposed to the ultraviolet rays, a small amount of dark brown material precipitated; this material formed colloidal solutions in dilute sodium hydroxide solution, but was insoluble in dilute acid solution. Much of the soluble pigment of the irradiated solutions could be removed by alternate additions of strong sodium hydroxide and sulfuric acid solutions; or by extraction from strongly acid solution with butyl alcohol.

2. Irradiated neutral, acid, or alkaline solutions of phenylalanine were found in the present investigation to give positive Millon tests, indicating the formation of a phenol. When this test was carried out under standard conditions,[2] a precipitate (composed chiefly of unchanged phenylalanine) formed. Removal of this precipitate by centrifugation left a clear, red solution whose color closely resembled that of a tyrosine solution tested in the same way.

3. Irradiated solutions developed a faint, orange-red color when they were treated with the nitrite-molybdate reagent.[2] Since irradiated tyrosine solutions give a similar color with this reagent as a result of the formation of dopa,[3] it may be inferred that a portion of the phenol (tyrosine?) formed during the phenylalanine irradiation was converted to a catechol (dopa?).

4. Spiegel-Adolf[1] suggested that the characteristic odor produced by irradiating phenylalanine solutions might be due to the formation of phenylacetaldehyde, but she offered no chemical evidence in support of this guess. In the present investigation, it was found that irradiated solutions of the amino acid gave strongly positive Schiff tests. Moreover, the solutions responded to the phenylhydrazine test by the formation of an insoluble crystalline phenylhydrazone. Much of the characteristic odor appeared to be concentrated in an oily, ether-soluble material that floated on top of the irradiated solutions.

5. Dry phenylalanine powder was exposed to the rays for long periods of time (about 10 days). The irradiated material then was analyzed for phenylalanine by the Kapeller-Adler procedure.[4] This test indicated that at least 20% of the phenylalanine had been destroyed.

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[2] Arnow, L. E., *J. Biol. Chem.*, 1937, **118**, 531.

[3] Arnow, L. E., *J. Biol. Chem.*, 1937, **120**, 151.

[4] Kapeller-Adler, R., *Biochem. Z.*, 1932, **252**, 195.

## 13636 P

# A Humoral Control of Parathyroid Secretion.

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There is considerable circumstantial evidence that a low serum calcium is a stimulus for the parathyroid glands to produce more hormone. Parathyroid hyperplasia may be induced experimentally by low calcium diets.[1] Clinically, hyperplasia of parathyroid tissue may occur in association with chronic nephritis.[2] This may be a compensatory reaction to the hypocalcemia which is the result of a phosphate retention and consequent hyperphosphatemia: The present work was undertaken to determine the relationship of a low serum calcium to parathyroid secretion.

For the production of a hypocalcemia the method of Hastings and Huggins was used.[3] Small dogs were alternately bled maximally and immediately transfused with an equal volume of decalcified blood. The blood was decalcified by shaking the plasma with solid lead phosphate. Allowing 10 minutes to elapse between transfusions, the total serum calcium decreased to 5.5-7 mg % after about 14 transfusions, and in several dogs localized muscle fibrillations and tremors were seen. Twenty minutes after the last transfusion the serum calcium rose 1 to 2 mg in both the normal dogs and in dogs thyroparathyroidectomized just prior to the experiment. Subsequent samples—40 and 80 minutes after the last transfusion—showed no further rise in serum calcium. Such transfusion experiments were performed on 7 normal and 3 thyroparathyroidectomized dogs.

In a second series of experiments, large amounts of sodium oxalate (40 mg/kg) were injected intravenously into both normal dogs and into dogs thyroparathyroidectomized just prior to the oxalate injections. The serum calcium fell immediately after the injections to 5.5-7 mg %, but rose promptly in the normal dogs and within 1½ to 3½ hours returned to the normal level (9.5-11 mg %). Only a very slight rise in serum calcium (1-2 mg %) occurred in the parathyroprival dogs and there was no further change although the observations extended over a 7-hour period (see Fig. 1). Experiments

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[1] De Robertis, E., *Anat. Rec.*, 1941, **79**, 417.

[2] Longmead and Orr, *Arch. Dis. Childhood*, 1933, **8**, 265.

[3] Hastings, A. B., and Huggins, C. B., Proc. Soc. Exp. Biol. and Med., 1933, **30**, 458.

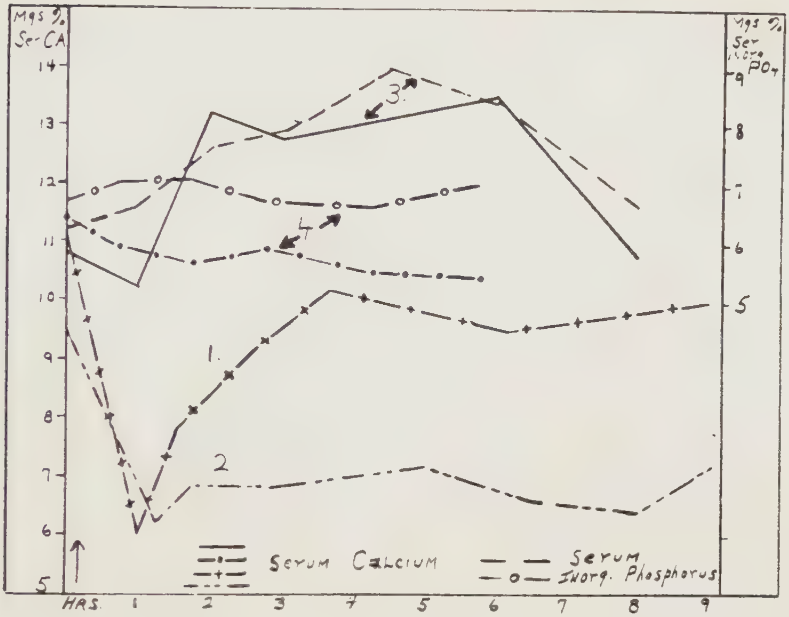

FIG. 1.

Serum calcium following I.V. injection of sodium oxalate into (1) normal dog and (2) dog thyroparathyroidectomized just prior to oxalate injection. Serum calcium and serum inorganic phosphorus following I.V. injection of decalcified blood parathyroid perfusate (3) and normal blood parathyroid perfusate (4).

of this nature were performed on 5 normal and 4 parathyroprival dogs.

A more direct experiment was performed as follows: The thyroid-parathyroid gland in a dog was isolated from the systemic circulation by tying appropriate vessels, leaving open only one artery (the superior thyroid) and one vein (the internal jugular into which several thyroid veins empty). The superior thyroid artery and the internal jugular vein were cannulated. Decalcified heparinized blood was perfused through the gland for 2 hours at a rate of approximately 3-4 cc per minute and the blood coming from the thyroid and parathyroids was collected. The plasma of this perfusate was then injected intravenously into a normal dog (anesthetized with sodium barbital) and the serum calcium and phosphorus changes were followed. A rise in serum calcium of from 1.6 to 4.5 mg occurred 1½ to 3 hours later. There was also an increase in serum inorganic phosphorus at this time, but the maximum elevation in phosphorus occurred somewhat after that of calcium (see Fig. 1). In one dog the rise in phosphorus was preceded by a fall. Alteration in serum inorganic phosphorus is not very significant unless correlated with urinary phosphorus. Unfortunately, simultaneous urine samples were not taken. This is being done in the present series of experiments. The calcium and phosphorus returned to normal in 4½ to 7 hours in 4 of the dogs but remained elevated in 3 dogs 7 hours after the injection.

When normal heparinized blood was perfused through the thyroid-parathyroid apparatus, the collected perfusate upon intravenous injection yielded no significant change in serum calcium and serum inorganic phosphorus. The maximum elevation in calcium was 0.6 mg % and in phosphorus 1.0 mg %. Eleven experiments involving the perfusion of both decalcified and normal bloods have been performed and are interpreted as evidence that a low serum calcium is a stimulus for the parathyroid glands to produce more hormone.

*Summary.* Preliminary investigations involving (a) the intravenous injection of sodium oxalate into normal and parathyroprival dogs and (b) the perfusion of decalcified blood through the thyroid-parathyroid apparatus and injection of the perfusate into normal dogs, indicate a humoral control (low blood calcium) of the parathyroid glands.

We wish to acknowledge gratefully the technical assistance of Dr. L. Eichelberger and Dr. E. DaCosta.

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### 13637

## Progesterone Effect on Pituitary Lactogen Content and on Mammary Glands of Ovariectomized Rats.*

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Selye[1, 2] has shown that the administration of progesterone alone is sufficient to induce the development of the lobule-alveolar system of the mammary glands of ovariectomized rats. Since the mammary glands of such rats showed no histological evidence of secretory activity, it was of interest to determine the influence of progesterone on the lactogen content of the pituitary gland.

Twenty-four sexually mature virgin rats were ovariectomized and divided into 4 groups on the basis of body weight. Beginning 14 days after ovariectomy 6 rats were injected subcutaneously daily for 10 days with 15 mg of progesterone,[†] in 0.5 cc of peanut oil, 6 were injected subcutaneously daily for 10 days with 33 gamma of estradiol benzoate,[†] 6 were injected subcutaneously daily for 10 days with 15 mg of progesterone + 33 gamma of estradiol benzoate, and 6 rats received no treatment. About 24 hours after the last injection the rats were killed and the pituitaries, adrenals, and thyroids (with the exception of the progesterone group) weighed. The 2 abdominal mammary glands were removed from each rat and one was studied as a whole mount and the other one in histologic section. The pituitary glands were assayed for their lactogen content by injecting the suspended tissue intradermally over the crop gland of common pigeons. Each group of pituitaries was assayed against each other group of pituitaries in 10 pigeons. Since the assay results for any one group of pituitaries were quite consistent the average of the 3 assays is presented.

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* Journal Series Paper of the New Jersey Agricultural Experiment Station, Rutgers University, Department of Dairy Husbandry.

[1] Selye, H., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 343.

[2] Selye, H., *Anat. Rec.*, 1940, **78**, 253.

Progesterone increased the lactogen content of the pituitary and this increase was brought about to a great degree by increasing the hormone concentration, since there was very little increase in pituitary size. Estrogen was more effective in augmenting the lactogen content than was progesterone, for estrogen not only increased the hormone concentration but it also caused pituitary hypertrophy. Progesterone + estrogen was slightly less effective in increasing pituitary lactogen content than was estrogen alone but slightly more effective than progesterone alone. Pituitary hypertrophy and an increase in hormone concentration were responsible for the increase in hormone content. The adrenal glands of the progesterone group and those of the progesterone + estrogen group weighed slightly less and those of the estrogen group slightly more than did those of the control group. During the 10-day injection period the control group made an average gain in body weight of 7 g, the progesterone group 17 g, and the progesterone + estrogen group 4 g. The estrogen group, on the average, lost 10 g in body weight. These results are summarized in Table I.

The mammary glands of the control rats consisted of an involuting duct system. The estrogen group's mammary glands consisted mainly of an extensive duct system with the ducts distended with secretory products. Progesterone treatment stimulated moderate

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[†] The progesterone and the estradiol benzoate were kindly supplied by Dr. E. Schwenk of the Schering Corporation.

TABLE I.
Effect of Progesterone in the Spayed Rat.

| Treatment (No. of rats) | Avg body wt beginning g | Avg body wt end g | Avg pituitary wt, mg | Bird units per pituitary gl. | Bird units per mg pituitary tissue | Avg adrenal wt, mg | Avg thyroid wt, mg |
|---|---|---|---|---|---|---|---|
| None (6) | 219 | 226 | 9.5 | 15 | 1.58 | 44.2 | 23.0 |
| 15 mg progesterone daily—10 days (6) | 217 | 234 | 10.1 | 27 | 2.67 | 39.9 | — |
| 33 $\gamma$ estrogen daily—10 days (6) | 215 | 205 | 14.9 | 38 | 2.55 | 48.3 | 24.5 |
| 15 mg progesterone + 33 $\gamma$ estrogen daily—10 days (6) | 215 | 219 | 15.1 | 33 | 2.19 | 41.0 | 27.1 |

lobule-alveolar development and only very limited secretory activity. The mammary glands of the progesterone + estrogen group showed extensive lobule-alveolar development and secretory activity somewhat greater than that observed in the progesterone group.

We were able to confirm Selye's[1, 2] observations of the positive effects of progesterone alone on the vagina and uterus of the spayed rat.

*Conclusions.* The daily injection of 15 mg of progesterone for 10 days into sexually mature spayed rats increased the lactogen content of their pituitary glands, caused no significant increase in pituitary weight, and induced moderate development of the lobule-alveolar system and limited secretory activity of the mammary glands. The daily administration of 15 mg of progesterone + 33 $\gamma$ of estrogen for 10 days caused extensive lobule-alveolar growth of the mammary glands. The secretory activity of the glands was somewhat greater than that observed following progesterone treatment. This treatment was less effective than estrogen but more effective than progesterone in increasing the lactogen content of the pituitary gland.

### 13638 P

# Effect of Androgen on Metabolism of Subcutaneous Corn-oil.*

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We have reported that when corn-oil is administered subcutaneously to mice it is seen to disappear from the site of injection more rapidly in the male than in the female. The sex difference in rate of utilization of oil is abolished by castration.[1] In this communication there are described preliminary studies of the influence of crystalline testosterone and estradiol benzoate upon this phenomenon.

Twenty-seven stock white male mice 4 to 5 months of age were castrated, and a series of injections of corn-oil was started 17 days later. The oil was given into the subcutaneous tissues of the back in doses of 0.1 ml semiweekly until a total of 1.0 ml was received by each mouse. Details of the method employed for administration of oil and its recovery from the site of application after a given time may be found elsewhere.[1] One-third of the animals was given estradiol benzoate (*ca.* 0.3 mg/mouse), one-third testosterone propionate (*ca.* 15 mg/mouse), the remainder served as controls. The hormones[†] were crystalline powders, suspended in dilute gum acacia just before use. They were injected subcutaneously over the anterior thorax in 2 equal doses, the first being given at the time of the initial injection of oil and the second 2 weeks later.

One week after the last injection of oil the animals were sacrificed. Table I indicates that the androgen-treated castrates utilized injected oil at a more rapid rate than the controls. They likewise consumed more food and gained somewhat less weight. Of the mice receiving estrogen, 5 died, probably from overdosage of hormone. The remaining animals of this group appeared to behave like the controls, but it cannot be said with finality that female sex hormone is unrelated to this phenomenon until the experiment can be repeated.

There is an analogy between these observations and some aspects of the clinical findings in obesity: changes in endocrine physiology

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* This work was carried out in part under a fellowship grant from the Commonwealth Fund.

[1] Turner, Joseph C., and Mulliken, Barbara, PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 598.

[†] Supplied through the courtesy of Dr. E. Henderson of the Schering Corporation, Bloomfield, N.J.

TABLE I.

| | Controls | Testosterone-treated* |
|---|---|---|
| Food g/mouse/day | 4.1 | 4.7 |
| Mean gain in wt, g | 4.2 | 3.4 |
| Ether-soluble material recovered from site of oil injection, mg. | | |
| | 366 | 113 |
| | 423 | 201 |
| | 452 | 281 |
| | 461 | 300 |
| | 463 | 325 |
| | 593 | 455 |
| | 598 | 464 |
| | 630 | 574 |
| | 690 | |
| Mean | 522 ± 25.3 | 339 ± 36 |
| $\sqrt{\frac{\Sigma d^2}{n-1}}$ | 112.7 | 150.9 |
| Diff. of Mean | 183 ± 44 | |
| x P E | 4.16 | |

*One animal died during the experiment.

such as castration or menopause are often followed by increase in body fat. Moreover, it has long been known that the metabolic rate of females is generally lower than that of males and the behavior of these mice affords another striking example of this peculiar dissimilarity of the sexes. The explanation of obesity based upon the law of conservation of energy forms, of course, a necessary part of any account of overall metabolism, but neglects biological relationships which operate at a different level. As examples, the recently discovered connection between androgens and nitrogen balance or the specific action of estrogens on the blood fat of birds may be mentioned. To account for the observed sex difference in rate at which injected oil is utilized any of several hypotheses might be adopted; we have been interested at this stage principally to determine whether the effect is mediated through the action of androgen or estrogen, or both. The data suggest that androgen may specifically increase the rate of utilization of depot fat. The method, through suitable modification, may prove useful in the study of this and similar problems as an index of the behavior of depot fat.

*Summary.* 1. The administration of testosterone propionate accelerates the rate of disappearance of corn-oil injected subcutaneously in castrate male mice. 2. It is suggested that this relationship is best explained by the hypothesis that androgen specifically increases the rate of utilization of depot fat.

We are indebted to Dr. S. C. Werner and Dr. E. G. Miller for many helpful suggestions.

## 13639 P

# Factors Influencing the Infectivity of Bacteriophage.

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When a mixture of virus and antiserum is titrated in animals it is the usual experience to find that the infectivity of the virus has been reduced but not abolished. By convention, one refers to such a mixture as representing so many units of neutralized virus, and so many units of active virus. In some instances this convention has been literally interpreted to reach conclusions probably not justified by the facts. In particular, decisions have been made regarding the reversibility of neutralization, and the heterogeneity of the viral population, on the insecure assumption that "neutral" and "active" viral particles in the mixture are different. It is not our purpose to question these conclusions, but to inquire into the validity of the underlying assumption.

The experiment described in Table I serves as an illustration of some of the difficulties inherent in an all-or-none hypothesis of neutralization. When the neutralization effected by a given exposure of bacteriophage to antiserum is evaluated by enumeration on three different media, 3 different results are obtained. With unsensitized phage, the media consistently fall into a definite order of effectiveness, but this order is profoundly changed after the phage has been exposed to antibody. Thus the medium representing the highest degree of "host-resistance"[1] to untreated phage, exhibits no unusual resistance after the phage has been sensitized. This experiment can be modified in many ways, but in each case it is evident that the active sensitized phage is qualitatively different from the unsensitized phage. Similar observations with the animal viruses[2] might be interpreted in the same way. The advantage of experiments with the simpler system lies in their susceptibility to further analysis, especially because the effects of uncombined antibody can be excluded.

The coliphage used (P9H) is weakly active in the absence of rather high concentrations of univalent cations. This is at least in part a result of poor adsorption to bacteria in the absence of salt. The effect of antibody is to increase the adsorbability in the presence or absence of salt. But antibody also inactivates phage, independently

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[1] Hershey, A. D., and Bronfenbrenner, J., *J. Gen. Physiol.*, 1941, **24**, 703.

[2] Bronson, L. H., and Parker, R. F., *J. Immunol.*, 1941, **41**, 269.

TABLE I.
Phage-Neutralization Tests with Different Media

| Medium | 0.08 M NaCl | 0.016 M NaCl | 0.08 M NaCl + dead bacteria |
|---|---|---|---|
| Unsensitized phage | 448 | 96 | 200 |
| Sensitized phage | 40 | 34 | 9 |

All counts shown express the phage-content of $10^{-7}$ ml of the same preparation of coliphage P9H. For sensitization, phage was exposed to the action of 1:5000 rabbit antiserum for 80 minutes at 37°C, and diluted beyond the range of activity of the serum. The counts were made simultaneously according to the method described in reference [1].

of the effect on adsorption. If the concentration of salt is low, antibody may either increase or decrease infectivity, depending on the balance between these two effects. If dead (or "resistant") cells are present, the effect of sensitization is to depress infectivity both by increasing adsorption to the dead cells, and by inactivating the phage.

We believe that these facts, as well as analogous results with animal viruses, can be better understood if it is assumed that the sensitized virus does not consist of a mixture of neutral and active particles, but that it represents an essentially uniform population of virus of altered infectivity. On this assumption, we have the general relation $P = (1\text{-}p_r\text{-}q)p_i$, where P is the infectivity expressed as the probability that any single specified viral particle will produce a lesion, $p_r$ is the probability that the particle will be accepted by a resistant cell, q is the probability that it will escape fixation (or be lost into the circulation) and $p_i$ is the probability that it will produce a lesion if fixed by a susceptible cell. If the susceptible cells are assumed to be uniform, $p_i$ is a simple probability; otherwise it expresses the average susceptibility of the cells not perfectly resistant. It is evident in what way salt, dead bacteria, and antibody in our experiment, diversely affect P by influencing the various terms on the right. In the case of other viruses, the analogous factors are only now being elucidated.[3, 4]

The available experiments do not establish the truth of the assumption made above. It is suggested merely that this assumption is reasonable, and should be applied to the results of infectivity-measurements before far-reaching conclusions are drawn.

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[3] Sprunt, D. H., *J. Exp. Med.*, 1941, **74**, 81.

[4] Friedewald, W. F., *J. Exp. Med.*, 1942, **75**, 197.

### 13640 P

## Comparison of Vitamin A of Liver Biopsy Specimens with Plasma Vitamin A in Man.*

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The liver, as the chief depot of vitamin A in the body, stores about 95% of it.[1, 2, 3] Therefore, clinical tests for the vitamin A status of the individual are best evaluated by comparing their results with the vitamin A content of the liver.[4] Consequently, in experimental animals, the blood vitamin A level has been compared with the vitamin A content in the liver. As a result of such studies, some[5, 6] considered the blood vitamin A level an index of the liver vitamin A stores and, hence, of the vitamin A saturation of the body. Others[7, 8] failed to find a correlation. In this study, we evaluated the plasma vitamin A level as a measure of the vitamin A saturation in man by comparing it with the vitamin A content of liver biopsies obtained at laparotomy.

Biopsies were taken from 34 patients with various upper abdominal diseases requiring operations. Some of them received supplements of vitamin A a few days before the operation. Blood specimens were drawn before, during, and after the operation. The liver specimens and the plasma were examined chemically (Carr-Price reaction) for vitamin A according to Josephs' method,[9] the liver being first hydrolyzed.[10] Furthermore, the vitamin A distribution of the liver was

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* This work was supported by a grant from the Committee on Scientific Research of the American Medical Association.

[1] Moore, T., *Biochem. J.*, 1931, **25**, 275.

[2] Baumann, C. A., Riising, B. M., and Steenbock, H., *J. Biol. Chem.*, 1934, **107**, 705.

[3] Popper, H., and Greenberg, R., *Arch. Path.*, 1941, **32**, 11.

[4] Nylund, C. E., and With, T. K., *Act. Med. Scand.*, 1941, **20**, 387.

[5] Horton, P. B., Murill, W. A., and Curtis, A. C., *J. Clin. Invest.*, 1941, **20**, 387.

[6] Lewis, J. M., Bodansky, O., and Haig, C., *Am. J. Dis. Child.*, 1942, **62**, 1129.

[7] Leong, P. C., *Biochem. J.*, 1941, **35**, 806.

[8] Brenner, S., Brookes, M. C. H., and Roberts, L. J., *J. Nutrition*, in press.

[9] Josephs, H. W., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 112.

[10] Skurnik, L., and Suhonen, P., *Z. Vitaminforsch.*, 1939, **8**, 316.

examined by fluorescence microscopy.[11, 12] Part of the liver specimen was submitted to routine histologic examination. On most patients, several liver function tests were also performed.

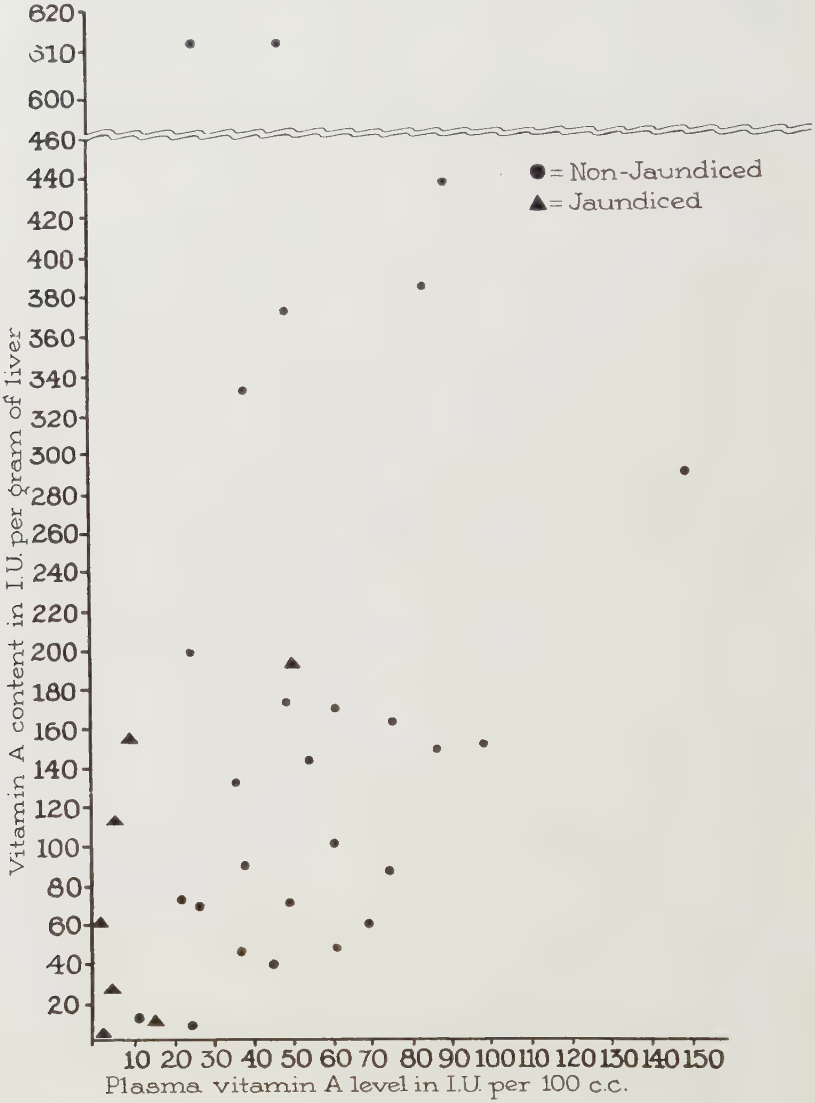

FIG. 1.

Plot of the plasma vitamin A level during operation against the vitamin A content of liver biopsies in man.

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[11] Querner, F. von, *Klin. Wchnschr.*, 1935, **14**, 1213.

[12] Popper, H., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 133.

Confirming previous studies[13, 14] great variation in distribution of vitamin A fluorescence (normally localized in Kupffer and liver cells) was found. In liver damage with or without jaundice the vitamin A fluorescence was reduced. The vitamin A fluorescence agreed fairly well with the amount of liver vitamin A determined chemically. In livers with high vitamin A content the Kupffer cells were especially rich in fluorescence.[3]

No parallelism, however, was evident between the plasma vitamin A level during operation and the liver vitamin A content as determined chemically and histologically. (Fig. 1.) In the majority of cases, no change in the plasma vitamin A level during operation was noted. Different vitamin A concentrations coincided with similar blood levels, *e. g.*, a plasma level of 50 I.U./100 cc was found in subjects with liver vitamin A content between 40 and 612 I.U./g. Plasma levels around 10 I.U./100 cc coexisted with liver concentration up to 160 I.U./g. However, the concentration of vitamin A in the liver was never low in subjects with plasma vitamin A above 80 I.U./100 cc. Low plasma levels of jaundiced patients coincided either with low or with average liver vitamin A.

These results suggest that the plasma vitamin A level indicates the vitamin A store in the liver only insofar as a high plasma level excludes a low liver storage. A low plasma level does not necessarily prove low liver storage. Apparently the vitamin A level of the blood fluctuates more readily and is sooner depleted than the vitamin A stores of the liver. Whether difficulties in release of vitamin A from the liver account for these discrepancies between plasma and liver vitamin A is being investigated.

*Summary.* The vitamin A content of biopsy specimens of human livers determined chemically and by fluorescence microscopy was compared with the plasma vitamin A level. No parallelism was found between liver and plasma vitamin A, except that subjects with high blood levels had at least an average liver storage.

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[13] Popper, H., *Arch. Path.*, 1941, **31**, 766.

[14] Meyer, K. A., Popper, H., and Ragins, A. B., *Arch. Surg.*, 1941, **43**, 376.

## 13641

# Assay of Adrenals for Lactogenic Hormone.*†

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Brownell, Lockwood and Hartman[1] postulated the existence of a specific lactation factor in the adrenals, "cortilactin," which they claimed was essential for normal lactation and separate from the life-sustaining factor. This material was separated from cortin by chilling out between 3 and –12°C. Spoor, Hartman and Brownell[2] precipitated out "cortilactin" isoelectrically from 86% alcohol at pH 5.8, following the method used by Riddle, *et al.*,[3] for extracting lactogenic hormone from pituitaries. The pigeon was used as an assay animal for this material, and an increase in crop gland weight served as a measure of its effect. They claimed that their best adrenal extracts were 1/10 as potent as a purified preparation of lactogenic hormone which contained 6 International Units (I.U.) per mg.

Inasmuch as this laboratory has been engaged in a study of the lactogenic hormone for a number of years, it was of interest to determine whether the adrenals contained true crop gland proliferating potency. The following materials were assayed: eschatin (Parke-Davis)[‡] adrenal cortical extract (Upjohn),[‡] whole macerated rabbit adrenals, and isoelectric precipitates of beef, hog and rabbit adrenals prepared by the Riddle, *et al.*,[3] method. The fresh beef and hog adrenals were secured from a local packing house. Only the beef adrenals were demedullated before extraction. All these materials were assayed by the highly sensitive intradermal "micro" pigeon test,[4, 5, 6] which enables one to measure quantitatively 1/160 I.U. of lactogenic

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[*] Aided by a grant from the Committee on Scientific Research of the American Medical Association.

[†] Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 816.

[1] Brownell, K. A., Lockwood, J. E., and Hartman, F. A., Proc. Soc. Exp. Biol. and Med., 1933, **30**, 783.

[2] Spoor, H. J., Hartman, F. A., and Brownell, K. A., *Am. J. Physiol.*, 1941, **134**, 12.

[3] Riddle, O., Bates, R. W., and Dykshorn, S. W., *Am. J. Physiol.*, 1933, **105**, 191.

[‡] The two commercial adrenal cortical extracts were kindly furnished by the Parke-Davis and the Upjohn companies.

[4] Lyons, W. R., and Page, E., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 1049.

[5] Bergman, A. J., Meites, J., and Turner, C. W., *Endocrinology*, 1940, **26**, 716.

[6] Meites, J., Bergman, A. J., and Turner, C. W., *Endocrinology*, 1941, **28**, 707.

hormone. This "micro" assay technic, which is based upon an observed proliferation response in the crop gland rather than on an increase in crop gland weight, has made it possible to assay the minute amounts of lactogen present in the urine of women and men,[4, 7] goats,[8] and in the blood[9] and placentae[10] of rabbits.

It is obvious from the data (Table I) that the various adrenal preparations used in this experiment did not contain crop gland proliferating activity. The 3 positive responses which were obtained can be attributed to the occasional proliferations which are sometimes observed in untreated pigeons, presumably because they had recently been incubating eggs. In the case of the isoelectric precipitates of beef, hog and rabbit adrenals, injections were made only over one crop gland of each pigeon, leaving the other crop gland as a control. Regardless over which side the injections were made, the right crop glands weighed approximately 30-40% more on the average than the left crop glands. The same weight difference was found in 10 uninjected pigeons, indicating that no increase was obtained in crop gland weight.

It is difficult to account for the increase in crop gland weight which was obtained by Spoor, *et al.*,[2] with their "cortilactin," although it is possible that substances other than lactogen may produce increases in crop gland weight. In a thorough study[3] involving 344 common

TABLE I.
Adrenal Substances Assayed for Lactogenic Activity in Common Pigeons.

| Material | Amount of extract injected per bird | No. birds | Crop gland response |
|---|---|---|---|
| Eschatin (Parke-Davis) | 10 dog units (.4 cc) | 10 | 0 +, 10 — |
| Eschatin (Parke-Davis) | 25 " " (1 ") | 30 | 2 +, 28 — |
| Adrenal Cortical Extract (Upjohn) | 50 " " (1 ") | 10 | 0 +, 10 — |
| Whole Rabbit Adrenal | 60 mg | 10 | 0 +, 10 — |
| Whole Rabbit Adrenal | 100 " | 10 | 0 +, 10 — |
| Rabbit Adrenal Extract | equiv. 1.5 g fresh material | 10 | 0 +, 10 — |
| Hog Adrenal Extract | equiv. 0.7 g fresh material | 10 | 0 +, 10 — |
| Beef Cortical Adrenal Extract | equiv. 6.0 g fresh material | 10 | 1 +, 9 — |
| Beef Cortical Adrenal Extract | equiv. 11.0 g fresh material | 5 | 0 +, 5 — |

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[7] Meites, J., and Turner, C. W., *J. Clin. Endocrinology*, 1941, **1**, 918.

[8] Hurst, V., Meites, J., and Turner, C. W., *J. Dairy Sci.*, 1941, **24**, 499.

[9] Meites, J., and Turner, C. W., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 190.

[10] Turner, C. W., and Meites, J., *Endocrinology*, 1941, **29**, 165.

pigeons, McShan[11] found that an increase in crop gland weight often did not indicate a positive lactogenic response, since such glands were visibly unproliferated. On the other hand, some crop glands which were visibly proliferated weighed no more than the crop glands of normal, uninjected pigeons. Since the proliferation of the crop gland mucosa, forming "crop milk", is a specific reaction to lactogenic hormone, it would seem that an assay method based upon such a response would be more accurate than one based on an increase in crop gland weight, which may not be specific for lactogenic hormone.

On the basis of the findings reported here, in which several types of adrenals were extracted by the same method used by Spoor, *et al.*, for "cortilactin", and despite the fact that a pigeon assay method at least 160 times as sensitive as that employed by the latter workers was used, it is concluded that the adrenals do not contain crop gland proliferating activity and therefore no lactogenic hormone.

*Summary.* Two commercial adrenal cortical extracts, whole untreated rabbit adrenals, and isoelectric precipitates of beef, hog and rabbit adrenals were assayed for lactogenic hormone by the sensitive intradermal "micro" pigeon crop gland technic. None of the adrenal compounds were found to contain lactogen.

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### 13642

## Synergism Between Thyrotropic and Growth Hormones of Pituitary. Body Weight Increase in Hypophysectomized Rat.*

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*From the Institute of Experimental Biology, University of California, Berkeley, California.*

It has been shown that the body weight gain stimulated in rats by alkaline extracts of beef anterior piuitary could be increased by adding relatively small amounts of thyroxin.[1, 2] At these dose

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[11] McShan, W. H., Ph.D. Thesis, 1936, University of Mo.

* Aided by grants from the Research Board of the University of California and from the National Research Council Committee on Research in Endocrinology. Assistance was rendered by the Work Projects Administration, Official Project No. 265-1-08-80, Unit A-5.

[1] Smith, P. E., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 1252.

[2] Evans, H. M., Simpson, M. E., and Pencharz, R. J., *Endocrinology*, 1939, **25**, 175.

levels, thyroxin, when given alone, caused only an insignificant body weight increase.[2] Because thyrotropic hormone is usually present in partially purified growth hormone preparations, it is very important in the interpretation of growth hormone assays of such fractions to know whether thyrotropic hormone exerts a similar synergic action, since such action might obscure the result. For this reason, in the present investigation, purified thyrotropic hormone was added to purified growth hormone, and the body weight increase elicited in rats by injection of the two hormones compared with that induced by the same amount of either hormone when given alone.

Hypophysectomized rats were used, in order to exclude synergic or antagonistic effects of other pituitary principles. Female rats were hypophysectomized at 26-28 days of age, and injections were begun approximately 10-15 days after operation. The average body weight at onset of the injections was 67 g. Animals were included in the results only after evidence for completeness of the operation had been obtained.[3] The rats were allowed to eat *ad libitum* McCollum's "diet I" moistened.[3] The hormones were administered daily for 15 days (13 injections), in experiments I, II and IV intraperitoneally, in experiment III subcutaneously. Both ways of application gave similar results in body weight gain as observed previously.[3]

In order to minimize effects of other active principles, it was considered important to use preparations of each of the two pituitary hormones, as free as possible of other "contaminating" hormones. The growth hormone preparations, obtained by a procedure described elsewhere[4] had an average minimum effective dose in hypophysectomized rats of 30 $\mu$g (daily dose). Follicle-stimulating, interstitial cell-stimulating, adrenocorticotropic, or thyrotropic hormones were not demonstrable in hypophysectomized rats when the preparation was given at a total dose of 5 mg (10-day test). Only when a total dose of 10 mg was given to squabs (4-day test), was a trace of thyrotropic activity found in one of the growth hormone preparations used. No lactogenic activity was demonstrable at a total dose of 20 mg (systemic crop test in squabs, daily subcutaneous injections for 4 days). The thyrotropic hormone preparations[†] [5] had a minimal effective dose of approximately 10 $\mu$g (daily dose) when assayed in hypophysectomized rats (4-day test). Small amounts of interstitial cell-stimulating and growth-promoting activity were demonstrable in this preparation when tested in hypophysectomized rats at 5 mg total dose. However, it is questionable if the growth-promoting activity of these preparations should be attributed to a contamination with growth hormone, since thyroxin causes similar initial insignificant body weight increases when given to hypophysectomized rats at doses which raise the metabolic rate to approximately the same level.

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[3] Marx, W., Simpson, M. E., and Evans, H. M., *Endocrinology*, 1942, **30**, 1.

[4] Fraenkel-Conrat, H. L., Meamber, D. L., Simpson, M. E., and Evans, H. M., *Endocrinology*, 1940, **27**, 605. Modifications of the procedure will be published in the near future.

[†] Kindly supplied by Jane Fraenkel-Conrat.

[5] Fraenkel-Conrat, J., Fraenkel-Conrat, H. L., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, 1940, **135**, 199.

The animals were divided in 4 groups, as indicated in Table I, which summarizes the results of 4 experiments. Both hormones were given at the same dose level of 80 μg daily.‡ One group of rats, (a), received both growth and thyrotropic hormones; another, (b), growth hormone alone; a third, (c), thyrotropic hormone alone; the last group, (d), served as control. In order to estimate the statistical significance of the results, standard errors were calculated according to Fisher's procedure for small samples.[6] From the observed body weight increases, growth hormone potencies were calculated for the experimental groups, using a standard curve for the relationship dose/response as basis for the computation.[3]

When given alone, thyrotropic hormone caused only an insignificant body weight increase, far less than that given by the mini-

TABLE I.
Synergistic Action of Thyrotropic Hormone with Growth Hormone on Body Weight Increase in the Hypophysectomized Rat.

| Hormones injected | No. of rats | Body wt gain in 15 days, g | Growth hormone potency, $[GU]^{15}_{H.R.}$* |
|---|---|---|---|
| (a) Growth and thyrotropic hormones combined | 28 | 31.8 ± 1.7 | 8.5 |
| (b) Growth hormone alone | 21 | 22.1 ± 3.2 | 2.4 |
| (c) Thyrotropic hormone alone | 29 | 5.9 ± 1.5 | (0.3) |
| (d) Controls | 13 | 2.5 ± 1.5 | — |

* $[GU]^{15}_{H.R.}$ = growth hormone units, 15-day test in hypophysectomized rats.[3]

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‡ Only in the fourth experiment was thyrotropic hormone administered at a dose level of 120 μg daily. When given in these amounts, thyrotropic hormone raises the metabolic rate in the hypophysectomized rat significantly, but not to normal levels; *e.g.*, with a dose of 80 μg of thyrotropic hormone, the oxygen consumption is increased from 126 to 143 $1/m^2/24$ hours (H. L. Fraenkel-Conrat and V. V. Herring, unpublished).

[6] Arkin, H., and Colton, R. R., *Outline of Statistical Methods*, 4th ed., New York, 1939.

mal effective dose for growth hormone—the "growth hormone unit".[3] Growth hormone, given alone, induced about 22 g growth during the experimental period. However, when, at the same dose levels, the 2 hormones were given in combination, a body weight gain of approximately 32 g resulted. Expressed in growth hormone units, this represents approximately a 3.5-fold increase in growth-promoting potency above that given by growth hormone alone. This result was achieved by adding a preparation which by itself is practically without effect on body weight. Thyrotropic hormone, therefore, parallels thyroxin in its synergic effect with growth hormone.

The mechanism of this synergism is not known at present. In the hypophysectomized rat, purified growth hormone induces deposition of tissue substance not only on account of an increased food intake, but also as a consequence of a better utilization of the consumed food.[7] Thyroxin also is known to increase food intake and to stimulate absorption from the intestine.[8] Therefore, it is possible that when thyrotropic hormone is given simultaneously with growth hormone, one or both of the mentioned phases of metabolism on which the action of growth hormone is based, may be stimulated to such an extent that a synergic effect results on the final phase of the growth hormone action—the deposition of tissue substance.

It is obvious why the body weight results reported here are of special interest for the interpretation of bioassays of pituitary growth hormone preparations, where the body weight gain is used as measure of growth hormone activity. When in the course of the purification, thyrotropic hormone is removed from a growth hormone preparation, the resulting product may appear to have a lower growth hormone potency, although it actually may have a higher growth hormone concentration and represent a "purer" growth hormone preparation than the starting material.

*Summary.* Purified pituitary growth hormone, when given in combination with purified pituitary thyrotropic hormone, is markedly increased in its capacity to stimulate body weight gain in the hypophysectomized rat. Since the thyrotropic preparation, given alone at the same dose level, is almost free of intrinsic growth-promoting potency, this action must be considered more than additive, and would appear to constitute a true synergistic effect.

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[7] Marx, W., Simpson, M. E., Reinhardt, W. O., and Evans, H. M., *Am. J. Physiol.*, 1942, **135**, 614.

[8] Russel, J. A., *Am. J. Physiol.*, 1938, **122**, 547.

### 13643

# Influence of Methyltestosterone on Metabolism of Normal, Castrate and Thyroidectomized Rats.

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From the general physiological action of sex hormones in eunuchs, consisting in the restoration of the low resilience of a sluggish organism to normal tonus, one would expect *a priori* a certain influence on the metabolic rate. Whether or not hypermetabolic levels could be reached by such treatment was less predictable. Eunuchoids usually give metabolic figures not as low as those found in myxedema, the lowest values reported being −30 and −24.[1, 2] Treatment with moderate doses of methyltestosterone by Byron and Katzen[3] did not result in metabolic rates definitely above normal, and Sandiford, Knowlton and Kenyon[4, 5, 6] obtained in eunuchoids with testosterone propionate (25 mg 3 to 7 times weekly) only moderate metabolic increases, if any at all, but no changes in a normal individual. Only McCullagh, *et al.*,[1, 2] reported definite hypermetabolic rates after prolonged treatment with large doses of methyltestosterone. Eidelsberg and Ornstein[7] found the metabolism not altered by testosterone propionate but the sensitivity to thyroid increased. Experiments with androgens in castrate dogs by Kochakian and Murlin[8, 9, 10] failed to show any metabolic increment, except in one animal after 3-4 weeks' treatment. A supplementary investigation in animals seemed desirable. The present paper reports the results obtained by treatment of rats with methyltestosterone.*

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[1] McCullagh, E. P., and Rossmiller, H. R., *J. Clin. Endocrinology*, 1941, **1**, 503.

[2] Jones, R., McCullagh, E. P., McCullagh, D. R., and Buckaloo, G., *J. Clin. Endocrinology*, 1941, **1**, 656.

[3] Byron, C. S., and Katzen, P., *J. Clin. Endocrinology*, 1941, **1**, 359.

[4] Sandiford, I., Knowlton, K., and Kenyon, A. T., *J. Clin. Endocrinology*, 1941, **1**, 931.

[5] Kenyon, A. T., Sandiford, I., Bryan, A. H., Knowlton, K., and Koch, F. C., *Endocrinology*, 1938, **23**, 135.

[6] Kenyon, A. T., Knowlton, K., Sandiford, I., Koch, F. C., and Lotiwin, G., *Endocrinology*, 1940, **26**, 26.

[7] Eidelsberg, J., and Ornstein, E. A., *Endocrinology*, 1940, **26**, 46.

[8] Kochakian, C. D., and Murlin, J. R., *Am. J. Physiol.*, 1936, **117**, 642.

[9] Kochakian, C. D., *Endocrinology*, 1937, **21**, 750.

[10] Kochakian, C. D., and Murlin, J. R., *J. Nutrition*, 1935, **10**, 437.

* We are indebted to Dr. William H. Stoner, The Schering Corporation, for the generous supply of this material.

Male rats, normal, castrate, thyroidectomized and thyroidectomized-castrate, were used. Castration was performed at 60 and 72 days of age, which means either immediately preceding or following maturation; thyroidectomy was always done at an age of 80 days, and in case of additional castration, this was done 20 days preceding or 20-25 days following thyroidectomy, the object being the discovery of any possible difference in the animals caused by the sequence of operations. The metabolic standardization was begun when the rats reached an age of 125-140 days and was carried on until satisfactory agreement was obtained on various days. The determinations were made in a closed Benedict apparatus, as described in a previous publication,[11] and the results were calculated in mg oxygen per hour per kilogram.

Taking, at random, from our record of over 1000 determinations those of 200 thyroidectomized rats, the average oxygen consumption was found to be 975 with a standard deviation of 80.7. Normal rats give an average value of $1500 \pm 100$. The methyltestosterone was given in daily subcutaneous injections (for this purpose a standard alcoholic solution was diluted with a calculated amount of water preceding use) mostly 500 $\gamma$ per dose, in some instances only 100 $\gamma$, in others 50 $\gamma$. The 500 $\gamma$ dose was proportionately in keeping with the massive doses used by Jones, *et al.* During the treatment the metabolism was determined at convenient intervals. At the end of the test period, the animal was treated with a standard dose of thyroid and the metabolic response observed. For this purpose a purified thyroid powder of 0.65% iodine content was used. From numerous tests we knew that this product, fed at a rate of 80 $\gamma$ per 10 g body weight daily for 3 days, causes in the thyroidectomized rat on the fifth day an increase in metabolic rate of 32-38% without affecting the heart rate materially. This potency was checked again in 2 thyroidectomized control rats. The metabolic change at the dose of 80 $\gamma$ per 10 g, was in the first rat from 1000 to 1360 mg oxygen, or 36%, in the second from 985 to $1360 = 38\%$. In normal rats 20 to 25 times that dose is required to produce the same effect, for which reason 1.6 mg was given for each 10 g weight to the normal as well as the non-thyroidectomized castrate rats. While in some of the animals of each group the treatment with the androgen was continued during the thyroid treatment, other animals were left without it for a certain length of time before the thyroid was given to show any influence which the androgen might have on the response to thyroid. The

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[11] Meyer, A. E., and Wertz, A., *Endocrinology*, 1939, **24**, 683.

TABLE I.

| No. of rats in group | History of group | Daily dose, $\gamma$ Me-testosterone | B.M.R. mg $O_2$ per hr per kg before and after days of treatment | | | | Treatment interrupted for days | B.M.R. after standard thyroid dose | % inc. over initial B.M.R. |
|---|---|---|---|---|---|---|---|---|---|
| | | | Bef. | 7 da. | 14-20 | 30 | | | |
| 4 | Thyroidectomy | 50 | 920 | 945 | 900 | | cont. | 1260 | +37 |
| 4 | at 80 days | 500 | 915 | 950 | 915 | | ’’ | 1240 | +36 |
| 4 | Thyroidectomy | 500 | 955 | 1110 | 1060 | | ’’ | 1320 | +38 |
| 4 | 80 days | 100 | 870 | 910 | 955 | | ’’ | 1275 | +46 |
| 2 | Castrated 105 days | 500 | 1030 | 1255 | 1125 | | 12 days | 1495 | +44 |
| 3 | Castrated 60 days | 500 | 930 | 1080 | 1020 | | cont. | 1370 | +47 |
| 3 | Thyroidectomy 80 days | 500 | 890 | 1000 | 1060 | | 11 days | 1275 | +43 |
| 4 | Normal | 500 | 1500 | 1500 | | 1550 | cont. | 1920 | +28 |
| 4 | Castrated 72 days | 500 | 1350 | 1300 | | 1320 | ’’ | 1840 | +36 |
| 4 | ’’ 60 ’’ | 500 | 1270 | 1310 | | 1295 | ’’ | 1725 | +36 |
| 4 | ’’ 60 ’’ | 500 | 1290 | 1355 | | 1355 | 10-18* days | 1800 | +40 |

*The B.M.R. after this period was 1235.

heart beat was determined routinely, but is not reported in Table I as no change was observed.

The metabolic rates before treatment with the androgen show that the castrate rat has a metabolic rate lower than the normal but higher than the thyroidectomized animal, which is in agreement with the experience in humans. Castration does not reduce the metabolic rate in thyroidectomized animals to still lower levels. Thyroidectomized rats gave no response to the androgen and neither were they influenced in their response to thyroid. Thyroidectomized castrates showed a moderate increase of the metabolic rate under the influence of methyltestosterone, usually, however, without exceeding the limits generally observed in the athyroid animals. This whole group gave an increased response to thyroid; the higher sensitivity to thyroid seems to be a consequence of the castration which is not altered by the methyltestosterone, since there was no difference between animals with continued or discontinued treatment. The sequence of the operations seems to have no influence on the response of the rats.

The normal rats treated with the androgen showed no deviation from the behavior of the untreated rat. The response to thyroid was, perhaps, slightly lower than usual, but normal animals do not give quite as uniform reaction in these tests as thyroidectomized rats, for which reason this lower value cannot be considered as significant.

The castrate rat, contrary to expectation, maintained an unchanged metabolic rate during the treatment. The final increase in metabolism after thyroid treatment was well within the limits of the normal rat, whether or not the methyltestosterone injections had been continued.

*Summary.* The oxygen consumption of castrate male rats is lower than that of normal, but higher than that of thyroidectomized rats. In castrate-thyroidectomized rats it is not lower than that found in non-castrate thyroidectomized rats. Treatment with methyltestosterone, within the conditions of these experiments, did not affect the metabolic rate of non-castrate animals, nor that of castrates. It caused a moderate increase in thyroidectomized-castrate rats. The latter rats showed also an increased sensitivity to treatment with thyroid, which, however, was neither caused nor influence by methyltestosterone.

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### 13644

## Effects of Gonadotropic Hormone in the Fish, *Xiphophorus helleri* Heckel.

FRANCIS MARSH BALDWIN AND MIN-HSIN LI.

*From the Physiological Laboratories, The University of Southern California, Los Angeles.*

The administration of mammalian gonadotropins from pregnancy urine and other preparations to a teleost fish, *Lebistes reticulatus,* has been reported by Berkowitz.[1] These substances stimulated the precocious development of male secondary sex characters, as well as the precocious but normally-patterned development of immature testes. The response of adult male *Lebistes,* if any, was by a decreased gonad size and an interstitial hypertrophy of the stroma. The latter phenomenon persisted only in some but not all of his fish when treatment was prolonged. He reported there was no effect on the secondary sex characters nor gonads of immature female *Lebistes.* The action of the hormones on mature females of this species was not mentioned. Saphir[2] found that injection of *prolan* from urine of pregnant women caused inconstant response of the "wedding dress" in male *Chromosomus erythrogaster.* Owen[3] produced this chromatophore reaction in 75% of male *Phodeus amarus* by employing the prolan in aquarium water, while he failed to induce the elongation of ovipositor in females so treated. Wholly negative results were reported by Koch and Scheuring,[4] Johnson and Riddle[5] in gonads of *Phoxinus* and *Salmo* by injection methods. Also similar results were reported by Landgrebe[6] on *Salmo* and eel. Still no general conclusions can be drawn in adult fishes of other species. In connection with some other phases of hormone administration, it was thought of some interest to study further the effects of a human chorionic gonadotropic hormone (pranturon*) on the adult female and adult male of a teleost fish, *Xiphophorus helleri* (swordtail).

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[1] Berkowitz, P., *J. Exp. Zool.*, 1941, **86**, 247.

Twenty virgin females were injected intraperitoneally for 10 to 20 weeks. Each fish received 25 I.U. (2.5 mg) gonadotropic hormone in .05 cc of distilled water weekly. A group of 10 virgin females were similarly injected with pure distilled water, and another group of 5 were left untreated. After 5 injections, a male-like gonopod was observed to develop on 4 of the treated females. Attempts at copulation simulating male behavior also occurred. One of these altered females was killed 60 days after the initial injection. Histological examination revealed a small unilateral maturing testis. A caudal sword developed in 2 of the 3 remaining females after 10 weekly injections. Gonads of these treated females show various stages of spermatogenesis with a moderate amount of interstitial cells. Secondary sex-coloration was observed in the completely sex-reversed fish.

While the masculinization of females by the administration of gonadotropic substances has been recorded in several higher vertebrates, *i. e.*, fowl,[7, 8] guinea pigs,[9] and rats,[10, 11, 12] the experimental induction of complete sex-reversal in fishes by the mammalian gonad-

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[2] Saphir, W., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 864.

[3] Owen, S. E., *Endocrinology*, 1936, **20**, 214.

[4] Koch, W., and Scheuring, L., *Zool. Anzeig.*, 1936, **116**, 62.

[5] Johnson, M. W., and Riddle, O., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 260.

[6] Landgrebe, F. W., *J. Exp. Biol.*, 1941, **18**, 162.

* By the courtesy of Dr. M. Gilbert of the Schering Corporation, Bloomfield, New Jersey.

[7] Domm, L. V., *Cold Spring Harb. Sympos. Quant. Biol.*, 1937, **5**, 34.

[8] Uotila, U. U., *Anat. Rec.*, 1939, **74**, 165.

[9] Papanicolau, G. N., and Falk, E. A., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 750.

[10] Greene, R. R., and Burrill, M. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 514.

[11] Bradbury, J. T., and Gaensbauer, F., *ibid.*, 1939, **41**, 128.

[12] Marx, L., and Bradbury, J. T., *Anat. Rec.*, 1940, **78**, 79.

otropic hormones has not been reported before. In the genetical gonochorist, *Xiphophorus helleri*,[13] Essenberg[14] has observed 2 cases of natural complete sex-reversal from female to male. The transformation of the female anal fin into a gonopod,[15-18] and a complete sex-reversal[17, 19] in *Xiphophorus* can be induced by the administration of androgenic substances. Essenberg[20] has pointed out that any adverse condition, be it extrinsic or intrinsic, acquired or inherited, which tends to reduce the capacity for hormone production beyond a certain limit may become the immediate cause of sex-reversal in the female *Xiphophorus helleri*.

Germ cells in various stages of the ovarian cycle were found in ovaries of both the unaffected and control females in our experiments. So far as could be observed, the gonadotropin of pregnant urine has no marked effect on the secondary sex characters in the unaffected female fish.

Twenty-eight sexually mature males were used in this experiment in which 8 served as controls. Twenty males were kept separately in 7 aquaria, each containing 1500 cc of water. The powdery gonadotropin was dissolved in the sterile diluent immediately before using. Seven hundred and fifty I.U. (75 mg) of the hormone were added weekly to each aquarium. From the surrounding water the hormone administered was absorbed by the gill tissue of the fish. All specimens were maintained at a temperature of 18° to 21°C during the experiments.

The experiments on adult males were conducted over a period of 4 months. Examination of testes from fish which were treated for 10 to 12 weeks as against controls show the gonadotropin had accelerated spermatogenesis; since the lumina of the sperm tubules were crowded with spermatophores, and distended with spermatozoa. It is indeed difficult to find spermatids and spermatocytes in these treated testes. *This accelerated effect of mature sperm formation* induced by the gonadotropin hormone was not noted by Berkowitz[21] in adult fish. In some instances, the testes appear to have undergone a state of precocity indicative of exhaustion, where some tubules even contained the desquamated epithelial cells and debris only. This gives evidence of hypotrophy and reduction in size. In all cases, a marked increase in interstitial tissue is evidently accompanied by vascular tissue growth, so that the testes are dense and compact in transection, as contrasted to those in controls. One may postulate that this interstitial hypertrophy of the stroma is a direct result of an attempt on the part of the testes to counteract the excess of the gonadotropins, and that this may terminate in sterility if the treatment is prolonged. It is well-known that many of the sex hormones have the power to suppress the hypophyseal functions.[22]

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[13] Witschi, E., Chapter 4, *Sex and Internal Secretions*, 1939, Williams and Wilkins Company, Baltimore.

[14] Essenberg, J. M., *Biol. Bull.*, 1926, **51**, 98; 1923, **45**, 46.

[15] Regnier, M. T., *Bull. Biol. de France et Belg.*, 1938, **72**, 385.

[16] Witschi, E., and Crown, E. N., *Anat. Rec.*, 1937, **70**, 121 (supplement).

[17,18] Baldwin, F. M., and Goldin, H. S., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 813; 1941, **46**, 283.

[19] Noble, G. K., and Borne, R., *Anat. Rec.*, 1940, **78**, 147 (supplement).

[20] Essenberg, J. M., *Biol. Bull.*, 1926, **51**, 98; 1923, **45**, 46.

[21] Berkowitz, P., *J. Exp. Zool.*, 1941, **86**, 247.

*Summary.* (1) This paper reports for the first time the possibility of complete sex-reversal in the adult female *Xiphophorus helleri* by administration of gonadotropic (human chorionic) hormone from pregnancy urine. (2) Gonadotropin when administered to the adult males of this species increases not only the amount of interstitial stroma (Berkowitz), but hastens spermatogenesis and brings about an exhaustion effect simulating a condition of sterility. (3) There were no significant effects on the secondary sex characters of the adult males and unaffected females under this treatment.

### 13645 P

## Exploration of a Method for Standardizing Hemorrhagic Shock.

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The urgency in developing standardized laboratory procedures for production of shock which will enable us to evaluate the efficiency of remedial measures with greater certainty is generally recognized. While no claim to such achievement can be made, we desire to report results which point to certain trails that ought to be explored more thoroughly.

Since the bulk of experimental work[1] strongly suggests that the progressive decline in arterial pressures is associated with reduced circulating volume, it appeared probable that the greatest hope of developing a standard laboratory procedure for producing shock lay in reducing the blood volume by bleeding. However, our studies showed that the dangerous factor in shock, *viz.*, its irreversibility, is not as definitely related to the percentile reduction in blood volume as it is to the degree and duration of hypotension which eventuates. As is well known, stray dogs differ greatly in regard to the percentile reduction in blood volume withstood before they develop a protracted low arterial pressure. Consequently, attempts to standardize procedures for creation of experimental hemorrhagic shock which are based on withdrawal of definite volume percent of the estimated blood are doomed to disappointment.

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[22] Moore, C. R., and Price, D., *Am. J. Anat.*, 1932, **50**, 13.

[1] Wiggers, C. J., *Physiol. Rev.*, 1942, **22**, 74.

We have sought to determine the degree and duration of posthemorrhagic hypotension which leads to a circulatory state that cannot be benefited more than temporarily by substantial infusions of the withdrawn blood (heparinized), and is generally accompanied by severe congestion, edema and hemorrhage of the upper intestinal mucosa.

*Results.* Fig. 1 shows graphically representative results selected from 75 experiments.

The data plotted in section A are from experiments in which mean arterial pressures were maintained by regulated bleeding between 46-60 mm Hg (average 50) for 1¾-4 hours. Irreversible shock never resulted. This emphasizes (1) the importance of distinguishing between posthemorrhagic hypotension and hemorrhagic shock, (2) the remarkable ability of tissues to withstand reduced blood

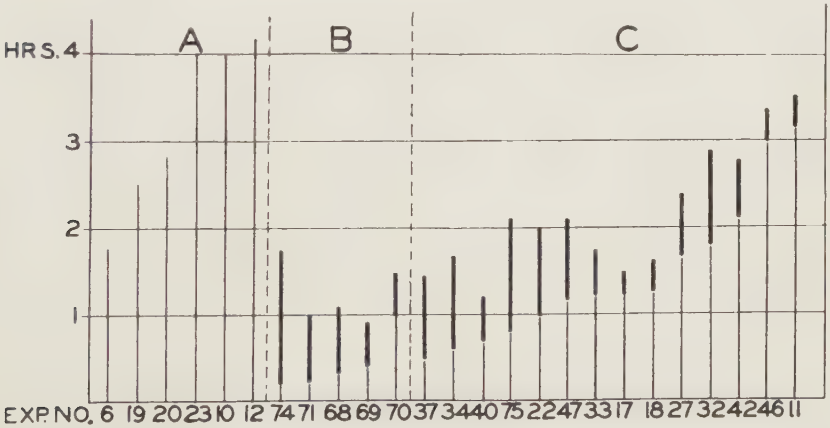

FIG. 1.

Diagrams showing intervals of moderate posthemorrhagic hypotension (narrow lines) and of extreme hypotension (broad lines) which proved effective and ineffective in causing irreversible shock. Discussion in text.

flow and (3) the impracticability of such a procedure for production of experimental shock.

The data plotted in Section B are from relatively fewer experiments in which an attempt was made to reduce arterial pressures to levels barely consistent with life (30-35 mm Hg). Regardless of the detailed methods of bleeding used, the compensatory reactions which come into play always result in brief preliminary periods of moderate hypotension before a drastic hypotension can be created. The tabular results show that a drastic hypotension (30-40 mm Hg) of an hour or more may not produce shock. In experiment 74, 105 minutes did not suffice.

The data of selected experiments plotted in Section C are from a group of animals in which varying intervals of moderate posthemorrhagic hypotension ($60 > 40$), followed by severe hypotension ($30 < 40$) after additional bleeding led to irreversible shock according to the criteria outlined. It is apparent that this group includes conditions which, in comparison with those of Section B, would not be expected to induce shock (*e. g.*, Exp. 40, 17, 18). Such variation in resistance or susceptibility of stray animals to hypotensive states precludes any scientific attempt at statistical analysis. However, a survey of our experiments shows that when the initial period of moderate, and the subsequent period of drastic hypotension were 90 and 45 minutes respectively, all animals tested developed irreversible shock. When these intervals were reduced to 60 and 30 minutes respectively, some animals did and others did not develop shock. It is possible that tests on a larger number of animals may establish minimum effective periods of moderate and severe hypotension between those ranges which will produce irreversible shock with reasonable safety. We suggest for ourselves and others who have access to cheaper animals for such studies, trials of 60-60 minutes and 60-45 minutes as minimum effective periods.

### 13646

# Is Human Placenta Permeable to Gonadotropic and Estrogenic Hormones?*

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The amount of gonadotropin and estrogen passing from the maternal to the fetal organism may be gauged by comparing the levels of these substances in the blood of the maternal organism and in the vein of the umbilical cord. The concentrations of hormones in the blood of the mother and the umbilical cord may, therefore, serve as a criterion of the permeability of the placenta to these hormones. The estrogen,[1, 2, 3] as well as the gonadotropin[1, 2] concentration in the blood of the umbilical cord has repeatedly been studied but without simultaneous hormone determinations in the maternal blood. In the following experiments, the estrogen and gonadotropin concentration of the umbilical vein, on the one hand, and that of the retro-placental serum, on the other, were quantitatively studied.

*Experimental.* Immediately after cutting the umbilical cord the arteries were clamped and the fetal blood flowing from the umbilical vein (about 20 cc) collected in a test tube. The retro-placental (maternal) blood was obtained during the expulsion of the placenta. After coagulation, the serum of both specimens was centrifuged.

*Determination of Gonadotropin.* The sera were 3 times vigorously shaken with ether. The small amounts of ether which remain in the serum were removed by allowing the sera to stand in the open air for an hour. These sera contained no free estrogen, as shown by control determinations in castrated female mice, and each were tested on 8 infantile female albino rats for their gonadotropin content. The quantity of serum to be tested was given to the animals in 6 subcutaneous injections over a period of 48 hours. From the 60th to the 120th hour after the experiment was begun, vaginal smears were taken; after this time, the animals were sacrificed and uteri and ovaries inspected. We considered 1 RU as that quantity of hormone which, with the above technic, produced full vaginal

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* Aided by a grant of the Rockefeller Foundation, New York.

[1] Zondek, B., *Hormone des Ovariums und des Hypophysenvorderlappens*, Springer, Berlin, 1st ed., 1931, p. 200; Springer, Vienna, 2d ed., 1935, p. 356; Zondek, B., *cf.* No. 1; Bruehl, R., *cf.* No. 1.

[2] Bruehl, R., *Klin. Wchschr.*, 1929, **8**, 1766.

[3] Loewe, S., and Voss, H. E., *Klin. Wchschr.*, 1926, **5**, 1083.

TABLE I.

Comparison of the Gonadotropin Content of Retro-placental Blood and Blood of the Umbilical Vein.

| Case No. | Retro-placental serum, RU | Serum from the umbilical vein, RU | Proportion, % |
|---|---|---|---|
| 1 | 20,000 | 300 | 1.5 |
| 2 | 20,000 | 500 | 2.5 |
| 3 | 15,000 | 500 | 3.3 |
| 4 | 15,000 | 500 | 3.3 |
| 5 | 20,000 | 300 | 1.5 |
| 6 | 15,000 | 1500 | 10.5 |
| 7 | 20,000 | 2000 | 10.5 |
| 8 | 10,000 | 1250 | 12.5 |
| 9 | 20,000 | 300 | 1.5 |
| 10 | 5,000 | 500 | 10.5 |
| Avg | 17,000 | 745 | 4.38 |

cornification. In our rats 1 RU is equivalent to 1/3 to 1/6 IU.

Our results are seen in Table I.

As shown in Table I, the serum of the umbilical vein invariably contained less gonadotropic hormone than the serum of the retro-placental blood. The maximum ratio (Case No. 8) was 12.5%, the minimum ratio (Cases Nos. 1 and 9) 1.5%; on an average the ratio was 4.38%.

*Determination of Estrone.* The freshly drawn sera were tested for content of free estrogen on 8 castrated female mice each. The dose to be tested was given to the animals in 6 subcutaneous injections over a period of 48 hours. From the 48th to the 96th hour vaginal smears were taken, and 1 MU was considered that quantity of estrogen which, with this technic, produced full vaginal cornification (Allen-Doisy test). In our mice 1 MU is equivalent to 2 IU. The results are given in Table II.

TABLE II.

Comparison of Estrogen Content of Retro-placental Blood and Blood from the Umbilical Vein.

| Case No. | Retro-placental serum, MU | Serum from the umbilical vein, MU | Proportion, % |
|---|---|---|---|
| 1 | 1500 | 400 | 27 |
| 2 | 1200 | 800 | 66 |
| 3 | 800 | 800 | 100 |
| 4 | 1500 | 1500 | 100 |
| 5 | 1200 | 400 | 33 |
| 6 | 1250 | 1000 | 80 |
| 7 | 1000 | 400 | 40 |
| 8 | 1500 | 1500 | 100 |
| Avg | 1243 | 850 | 68 |

As is seen from Table II, in 3 cases (Nos. 3, 4, and 8) the concentration of estrogen in the umbilical vein was the same as in the retro-placental blood of the mother. In the other cases the concentration of estrone in the umbilical vein was lower; the minimum ratio was 27%; the maximum ratio 80%; and the average 68%.

*Results.* The above experiments show that the concentration of gonadotropin in the blood serum of the umbilical vein is far lower than that of the corresponding retro-placental blood serum. Thus it appears that the placenta allows the passage of gonadotropic hormone only to a very limited extent. Goodman and Wislocki[4] obtained similar results in their experiments on animals. They injected gonadotropin in pregnant rabbits or cats, but were unable to recover the injected substance from the amniotic fluid.

Determination of the estrogen content of the retro-placental and umbilical serum showed the concentration to be more or less equal in either case. The differences were at least not as pronounced as those in gonadotropin content. The placenta is, therefore, to a great extent permeable to estrogenic hormone.

*Summary.* The free estrogen and gonadotropin content of the serum from the umbilical vein and the corresponding maternal retro-placental serum were studied. The maximum concentration of gonadotropin in the serum of the umbilical vein was 12.5%; the minimum 1.5%; and the average 4.38% of that in the retro-placental blood.

The concentration of estrogen in the umbilical vein was either equal or only slightly different from that of the retro-placental blood. It averaged 68% of that in the retro-placental blood.

### 13647

## Ornithosis (Psittacosis) in Pigeons and Its Relation to Human Pneumonitis.

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In a previous paper,[1] it was reported that a psittacosis-like virus had been isolated from the lung of a patient who had been exposed to a flock of racing pigeons *(Columba livia)*. Nineteen, or 63%, of the 30 suspected birds gave specific complement-fixation reactions in dilutions of from 1:8 to 1:256 (Table I). A virus, indistinguishable from that of the patient, was isolated from the spleens and livers of 4 pigeons which had given a positive, and from 1 pigeon which had given a negative serum reaction. This observation was the prelude to the recognition of the nation-wide existence of a psittacosis-like infection in pigeons—in the future to be described as *"Ornithosis."* At least 10 human atypical pneumonias with 2 deaths [California 4 (2 deaths), New York 4, Minnesota 1 and Boston 1] have been traced to exposure to the different species of pigeons exclusively.

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[4] Goodman, L., and Wislocki, G. B., *Am. J. Physiol.*, 1933, **106**, 323.

[1] Meyer, K. F., *Schweiz. med. Wchnschr.*, 1941, **71**, 1377.

*Survey of Pigeon Lofts and Isolation of Virus.* In order to secure comparative data relative to the extent of *ornithosis* in lofts and to have available suitable birds for experimental tests, random samples of pigeons were secured from various places in California and other states of the Union. With the exception of 12 squabs from a loft whose old birds were virus-carriers, a universal existence of the infection was recognized with the aid of complement-fixation tests. The virus responsible for these reactions was isolated from the organs of non-reacting as well as of reacting pigeons. The pertinent data thus far obtained are summarized in Table I.

The extraordinarily high percentage of positive results will doubtless raise questions regarding the specificity of the serum-reactions. There is conclusive evidence that they are the result of a response to a psittacotic antigen. Pigeons whose serum is free from antibodies promptly generate them following the intramuscular injection or feeding of parrakeet-psittacotic or pigeon ornithotic viruses. Moreover, a flock of birds in which 60% of reactors had been demonstrated, were used as test animals for a bacterial biological product. At the end of the experiment they were bled again, and 95% of the sera gave positive reactions in the higher dilutions. This observation may correctly be interpreted as a reactivation of antibodies indicative of a previous contact or experience of the pigeons with the ornithotic antigen. The serum-reactions have been correlated with the carrier-state of the birds. The organs of 6 Visalia and 10 Hanford pigeons, the latter connected with a fatal infection, were individually tested in mice. From the Visalia pigeons 4 isolations were successfully obtained, 1 from a reacting, and 3 from non-reacting birds; from the Hanford pigeons 4 isolations were successfully obtained from the 7 reacting birds, and none from the non-reacting birds. No satisfactory explanation is as yet possible for the discrepancies. However, it is not unlikely that the positive complement-

TABLE I.

Complement-Fixation Reactions of Pigeon-Sera with Ornithotic Virus
Isolation of Virus.

California.

| Titer | San Leandro Old | Hayward Young | Hayward Old | Stock Old | Racing pigeons | Visalia | Pasadena I Source of fatal case Old ♂ | Pasadena I Source of fatal case Old ♀ | Pasadena II Old | Hanford Source of fatal case Old | El Monte Source of case ♂ | El Monte Source of case ♀ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 6 | 12 | 6 | 6 | 1 | 4 | 3 | 8 | (9) | 3 | 4 | 2 |
| 1:2 | | | | | | | | | | | | 1 |
| 1:4 | 1 | | | | | | | | | | 1 | |
| 1:8 | 1 | | 1 | | | | | 1 | | | | 1 |
| 1:16 | 1 | | 1 | 6 | | | | | | | 1 | |
| 1:32 | 1 | | 1 | 1 | 1 | | 4 | 2 | 2 | 2 | | |
| 1:64 | 2 | | | 1 | 2 | | 4 | | 2 | 3 | | |
| 1:128 | | | 2 | 2 | | 2 | 3 | 4 | | | | 2 |
| 1:256 | | | 1 | | | | | 1 | | 2 | | |
| Total | 12 | 12 | 12 | 16 | 4 | 6 | 30 | | 13 | 10 | 12 | |
| % reactors | 50% | 0 | 50% | 62% | 75% | 33% | 63% | | 30.7% | 70% | 42% | |
| Isolation of virus | Not attempted | Not attempted | | 1 | 1<br>1 C.F.— | 4<br>1 C.F.+<br>3 C.F.— | Incomplete<br>5<br>4 C.F.+<br>1 C.F.— | | Incomplete<br>1<br>1 C.F.— | 4<br>4 C.F.+ | 3<br>1 C.F.+<br>2 C.F. | |

United States.

| Titer | Indiana Old | Iowa Farm A | Iowa Farm B | Iowa Farm C | N. Y. City Old | Albany, N.Y. | Brooklyn Source of case | South Carolina Young | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 10 | 8 | 2 | 6 | 10 | 15 | 1 | 6 | |
| 1:2 | 2 | | | | | | | | |
| 1:4 | | 1 | | 1 | 5 | | | | |
| 1:8 | 4 | | | | 3 | | 1 | 3 | |
| 1:16 | 2 | | | | 3 | | 1 | 1 | |
| 1:32 | 2 | 1 | | | 4 | | | | |
| 1:64 | 2 | 3 | | 1 | 2 | | | 1 | |
| 1:128 | 2 | | | | 3 | | 1 | 1 | |
| 1:256 | 1 | | | | | | 1 | | |
| Total | 25 | 13 | 2 | 8 | 30 | 15 | 5 | 12 | Total |
| % reactors | 60% | 38% | 0 | 25% | 66% | 0 | 80% | 50% | 237 |
| Isolation of virus | Not tested | | Not tested | | 15 tested<br>3 C.F. + | 2+ | Incomplete | 1<br>1 C.F.— | 25 virus strains |

fixation reaction is merely indicative of a past or latent infection, and not necessarily of an active one. There still exists the other possibility that the virus may reside in organs other than the spleen, liver and kidneys which were used as inocula.

The two lots of pigeons offered certain advantages for successful isolations, since the birds were not simultaneously infected with *Salmonella typhi murium.* Concentrated organ-emulsions were well tolerated by the mice, and the ornothotic virus during its residence for 20 days in the spleens was sufficiently enriched so that the intracerebral transfer in the next passage produced lesions and microscopical findings; it was not necessary to filter the suspensions through a collodion membrane of over 400 m$\mu$ pore-width. Moreover, one of the Visalia pigeons, with a complement-fixation titer of 1:128++++, presented at necropsy a residual plastic pericardial exudate of recent origin, which showed suggestive pale intracellular plaques but no definite elementary bodies. A flake of the exudate suspended in a few drops of broth and inoculated intracerebrally in mice produced the typical ornithotic choriomeningitis, and in the next passage by intranasal injection a focal pneumonia.

*Parasitism of Ornithotic Virus.* The serological reactions and the isolation of 25 viral strains from a relatively small cross-country sample makes it clear that ornithosis of pigeons is probably ubiquitous. The agent possesses a highly adapted latent parasitism for pigeons which is somewhat similar to that of the psittacotic virus encountered in the parrot-species of the Australian regions. How it escapes the body of the birds is unknown, although suspicion is cast on the urine and the fecal droppings.

Infection appears to be acquired in the nests or at least in youth. Recovery from this early contact with the parasite ensures a lifelong relative resistance. But, since it is frequently associated with latency, the balance of the immunity may become disturbed in favor of the virus. That improper feeding may arouse these subclinical infections, in particular, that a thiamin-deficient diet may activate them, is beautifully illustrated by the observations of Pinkerton and Swank.[2] Likewise, the accidental crowding of a dozen pigeons in a poorly lighted, unsanitary cage in the Hooper Foundation induced frank clinical ornithosis and death of 6 birds. The nature of the illnesses and the necropsy findings were at first mistaken for pigeon-paratyphoid, since cultures yielded *Salmonella typhi murium.* The emaciated cadavers presented a fibrinous peritonitis and pericarditis, enlarged spleens (average 8 x 18 mm) with subcapsular hemorrhages, soft enlarged hyperemic livers, grayish kidneys, and catarrhal enteritis. The pneumonic foci reported for pigeon-paratyphoid were likewise present in 2 birds. Microscopical examinations of the serous exudate revealed, aside from the Gram-negative rods, an enormous number of bodies like those found in psittacosis. It is not unlikely that the relationship of the bacterial carrier-stage to the latent ornithosis is the same as that proven for the South American parrots.[3]

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[2] Pinkerton, H., and Swank, R. L., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 704.

*The Virus.* Morphologically and tinctorially, the *Microbacterium multiforme* of pigeon origin is indistinguishable from that originally obtained from Australian and South American parrots or American parrakeets, or from human infections traced to such sources. It is present in large numbers, free or in monocytic cells of avian or mammalian tissues, or in Zinsser-Fitzpatrick-Wei cultures. Organ suspensions, or cultures of these organisms produce in mice, ferrets and hamsters on intranasal injections, or in guinea pigs and monkeys on intratracheal injection, focal or lobular pneumonias of the same character as induced with the true psittacotic viruses. Even continuous passage through mice fails to enhance the pathogenicity, and fatal infections on intraäbdominal injections are rarely observed except upon the administration of overwhelming doses. This peculiar low pathogenicity for mice has likewise been noted by Coles[4] in connection with his studies of pigeons found infected with "psittacosis" in Johannesburg, Union of South Africa. However, recent observations indicate that occasionally a virus of greater mammalian pathogenicity may be encountered.

Parrakeets and ricebirds *(Munia oryzivora)* are fatally infected by feeding or by inhalation. Pigeons and an occasional dove *(Streplopelia?)* are clinically not affected by feeding or intramuscular injections, but continue to carry the virus in the organs for many weeks. Since Bedson and Western[5] failed to infect pigeons, it was generally believed that individual birds are refractory to the disease. In this connection, it is important to recall that doves *(Streplopelia decaocto* and *Streplopelia semitorquata)* may become infected spontaneously with the psittacotic virus of parrot or parrakeet origin when exposed in zoological gardens (Tomlinson[6]). It was noted in work done in coöperation with Dr. H. Pinkerton, that the ornithotic viruses quite regularly cause fatal meningitic infections, even in pigeons whose sera are strongly positive in the complement-fixation test; while the parrot- or parrakeet-psittacotic viruses, of either avian or human origin, induce these infections only in a few individual pigeons, and only when the viruses are recently isolated.

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[3] Meyer, K. F., and Eddie, B., *J. Infect. Dis.*, 1939, **65**, 234.

[4] Coles, J. D. W. A., *Onderstepoort J. Vet. Science and Animal Industry*, 1940, **15**, 141.

[5] Bedson, S. P., and Western, G. T., *Reports on Public Health and Medical Subjects*, N. 61, London, 1930, 84.

[6] Tomlinson, C. T., *Pub. Health Rep.*, 1942, **56**, 1073.

Crude antigenic analyses have shown no differences in the composition of the various strains, or differences between the psittacotic or ornithotic agents. These pigeon-viruses are indistinguishable from the "meningo-pneumonitis" virus of Francis and Magill,[7] which in comparative tests proved fatal to doves and pigeons on intracerebral, and to ricebirds and parrakeets on intramuscular injections.

*The Relationship of Pigeon-Ornithosis to Human Illness.* Epidemiologically, the evidence in its broader aspects grew more convincing as the observations became more complete. Due to unavoidable delays or late reporting, the complete transmission-chain, consisting of finding the virus in the patients and then in the birds, has not been established in every case. For example, a patient in New York with an atypical pneumonia and serum-reaction of 1:128+++ on the 16th day, produced sputum on the 19th day, from which a pigeon virus was isolated. He had a brief exposure to barnyard birds, including pigeons, in up-state New York. It was impossible to secure specimens of sera or cadavers of the birds.

However, the difficulties are in no way different from those experienced in connection with psittacosis due to exposure to parrots and parrakeets. A part of the chain depends on inductive evidence supported by a fairly dependable serological test. The early victims of ornithosis had either handled clinically diseased birds, or had been near or within lofts or barns in which they had been housed; the more recent histories fail to stress these observations. One would suspect by analogy with true psittacosis that pigeons in the active stage of infection discharge more virus and are more dangerous spreaders than the apparently healthy latent carriers. In the rural environment, or on the plazas and parks of towns and cities, large groups of people are constantly exposed to pigeons whose sera react in the complement-fixation tests. With such a vast reservoir of ornithotic virus available everywhere, one wonders why many sporadic atypical pneumonias (colloquially and wrongly diagnosed as "virus" pneumonias) are not in some way connected with these sources. An inquiry into the existence of subclinical or latent infections in certain groups would indeed be of interest. That they occur is attested by the observation that the pigeon-fancier, whose father died from ornithosis, had specific antibodies against the virus in his serum. Aerogenic contamination of the respiratory mucosa by the public in a great many civic places can be readily visualized. A patient may not have been cognizant of this exposure. Thus, the fortuitous isolation of a saprophytic ornithotic virus from nose- or throat-washings, in the course of an attack of influenza or a cold, might often happen.

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[7] Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, **68**, 147.

In the future, it appears highly desirable (a) that the sera of patients with atypical pneumonias, influenza and other respiratory infections, in the absence of specific bacterial agents, be tested for specific antibodies against the psittacotic or ornithotic virus, (b) that in the course of epidemiological investigations, the prolonged or even fleeting exposure to pigeons or barnyard birds like fowls and ducks be kept in mind, and (c) that the term psittacosis be limited to the human infections definitely proven to be of association with psittacine birds, while those due to contact with other birds be described as ornithosis of fulmar, pigeon, or chicken origin.

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### 13648

## Toxicity of Washings from *Hemophilus pertussis* for Mice.*

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While studying the toxic substance that can be obtained by growing *Hemophilus pertussis* in a liquid medium, as described by Wood,[1] we have observed that a substance with similar toxic properties can be obtained by simply washing the organisms of freshly isolated strains grown on a solid medium. After 48 hours of growth, the organisms are removed from the surface of Bordet's medium with a cotton swab and suspended in 0.85% NaCl solution. When density of the suspension has been adjusted to approximately 40 billion organisms per ml, the suspension is thoroughly mixed for 5 minutes with a pipet. The organisms are then sedimented in an angle centrifuge at a temperature of 4°C. After several periods of centrifuga-

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* Supported in part by a grant from the John and Mary R. Markle Foundation.

[1] Wood, M. L., *J. Immunol.*, 1940, **39**, 25.

TABLE I.
Toxicity of Saline Washings from *Hemophilus pertussis* for Mice.

| Test No. | Strain | No. of organisms washed in billions per ml | Dilution of washings* None | 1-2 | 1-4 | 1-8 | 1-16 |
|---|---|---|---|---|---|---|---|
| 1 | Jen. | 10 | 0/2† | 0/2 | 0/2 | | |
| 2 | ” | 20 | 4/4 | 0/4 | 0/4 | 0/4 | |
| 3 | ” | 30 | | | | 1/2 | 0/2 |
| 4 | ” | 40 | 4/4 | 4/4 | 4/4 | | |
| 5‡ | ” | 40 | 3/3 | 3/3 | 1/2 | | |
| 6§ | ” | 80 | 1/2 | 0/2 | 0/2 | | |
| 7 | ” | 100 | 2/2 | 2/2 | 2/2 | | |
| 8 | Kin. | 80 | | 3/4 | 2/4 | 0/4 | 0/4 |
| 9 | Ash. | 80 | | 4/4 | 2/4 | 0/4 | 0/4 |

*Dose = 0.2 ml given intravenously.

†The numerator indicates the number of mice that died in a group containing the number given in the denominator.

‡Same preparation as test 4, after 10 days storage at 4°C.

§Second washings from same organisms as test 4.

tion of an hour each, the supernatant fluid is clear but still contains a few organisms, as tested by culture. After from 6 to 8 such periods of centrifugation, however, a bacteria-free supernatant fluid can be obtained. Supernatant fluids so prepared have been lethal for 3-week-old Swiss mice when injected intravenously (Table I).

Our results show that the supernatant washings and the toxic substance obtained by the method of Wood[1] are lethal in comparable dilutions of 0.2 ml doses. The mice injected with sublethal doses

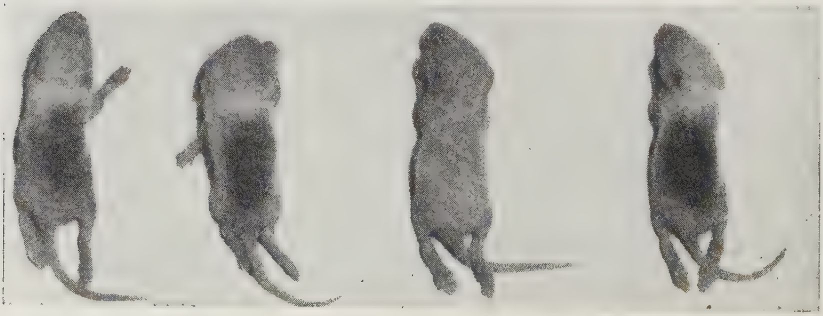
18 Hours After Injection I.D.
Dead
Dead
Killed
Dead
H. pertussis washings + globulin (living organisms)
H. pertussis washings + globulin (killed organisms)
H. pertussis washings + antitoxin
H. pertussis washings alone, control

Fig. 1.

Effects of refined antitoxic and antibacterial sera upon the skin-reacting factor in washings from *H. pertussis.* 0.05 ml of a 1-10 dilution of serum was mixed with 0.05 ml of undiluted washings and the mixture incubated for 30 minutes at 37°C before endermal injection.

showed an atrophy of the spleen and other pathological changes resembling those described by Wood. When 0.05 ml was injected endermally into 10-day-old mice, it produced a marked hemorrhagic necrosis at the site of injection and frequently killed the animal (Fig. 1). Both the lethal and the skin-reacting factors were destroyed by heating in a water bath at 54°C for 10 minutes. After 10 days of storage at 4°C, the lethal factor remained essentially unaltered in bacteria-free washings. A considerable amount of the toxic component was removed by passage through a Seitz filter. Neutralization experiments gave identical results with the toxic substance prepared by Wood's method and with our toxic washings.

It was observed that the lethal and skin-reacting factors were neutralized by a refined antitoxic serum, but not by either of 2 antibacterial-globulin solutions, one of which was prepared by immunizing rabbits with killed organisms, the other, with living organisms (Table II and Fig. 1). All of these findings closely resemble those obtained by Wood with her toxic substance and support her suggestion that the toxic factor in broth cultures may have been derived from the surface of the growing organism.[1] That surface-washings are not the only source of heat-labile toxin is well

TABLE II.
Neutralization of Toxic Washings by Refined Antitoxic and Antibacterial Sera.

| Dilution of serum | Sera tested* | | | Titration of lethal factor in washings | | |
|---|---|---|---|---|---|---|
| | A | B | C | Strain | Dilution | |
| 1-10 | 0/4 | 3/4 | | Jen. | 1-8 | 1/2 |
| 1-100 | 0/4 | 4/4 | | | 1-16 | 0/2 |
| 1-5 | | 4/4 | | Jen. | none | 2/3 |
| 1-10 | | 4/4 | | | 1-2 | 3/3 |
| 1-100 | 0/4 | 4/4 | | | 1-4 | 1/2 |
| 1-500 | 1/4 | | | | | |
| 1-10 | | 4/4 | 4/4 | Kin. | 1-2 | 3/4 |
| 1-100 | 0/4 | | 4/4 | | 1-4 | 2/4 |
| 1-500 | 0/4 | | 4/4 | | 1-8 | 0/4 |
| 1-10 | | | 4/4 | Ash. | 1-2 | 4/4 |
| 1-100 | | | 4/4 | | 1-4 | 2/4 |
| 1-500 | | | 4/4 | | 1-8 | 0/4 |

Serum A—*Pertussis* antitoxin (rabbit) obtained through the courtesy of Dr. A. L. Joyner of Lederle Laboratories, Pearl River, N.Y.

Serum B—Immune-globulin solution produced by immunizing rabbits with killed organisms, obtained through the courtesy of Dr. W. E. Bunney of E. R. Squibb and Co., New Brunswick, N.J.

Serum C—Immune-globulin solution produced by immunizing rabbits with living organisms (supplied by Dr. Bunney).

*0.2 ml of diluted serum was mixed with 0.2 ml of undiluted washings and the mixture incubated for 30 minutes at 37°C before intravenous injection.

known, because it has been obtained from extracts of disrupted, washed organisms.[2, 3] The toxic substance in washings from *H. pertussis* seems to be analogous to that obtained by Zinsser and by Parker[4] from a variety of other organisms and called by them the "X-substance."

In *H. pertussis* washings other components, such as a heat-stable factor, an agglutinogen, and derivatives from the culture medium, are no doubt present in varying amounts. That a specific soluble substance could be removed by washing the organisms with saline or water has been demonstrated by Miller.[5]

The chemical composition, the antigenic properties and the effect of the toxic washings upon blood leucocytes are interesting points now being studied.

*Summary.* A heat-labile substance, toxic for young mice has been obtained by washing suspensions of freshly isolated strains of *Hemophilus pertussis* with 0.85% NaCl solution. Both the lethal and the skin-reacting factors of the toxic substance can be neutralized by antitoxin but not by antibacterial rabbit sera.

### 13649

## Non-Specificity of Sulfonamides.

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(Introduced by N. Kopeloff.)

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The notion that individual members of the sulfonamide series are specific for certain bacterial species is widespread, although contrary to the most widely accepted hypothesis of the mode of sulfonamide action. The following experiments serve to clarify this point:

Staphylococci were grown on Knight's medium modified by Fildes, *et al.*,[1] and *Escherichia coli* on the asparagine-glucose-mineral

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[2] Evans, E. G., and Maitland, H. B., *J. Path. and Bact.*, 1937, **45**, 715.

[3] Flosdorf, E. W., and Kimble, A. C., *J. Immunol.*, 1940, **39**, 475.

[4] Zinsser, H., Enders, J., and Fothergill, L., *Immunity Principles and Application in Medicine and Public Health*, The Macmillan Co., New York, 1939, 78-101.

[5] Miller, J. J., PROC. SOC. EXP. BIOL. AND MED., 1937-38, **37**, 45.

[1] Fildes, P., Richardson, G. M., Knight, B. C. J. G., and Gladstone, G. P., *Brit. J. Exp. Path.*, 1936, **17**, 481.

TABLE I.
Concentrations of Sulfonamides Permitting ½ Maximum Growth.

| | Knight's medium | | MacLeod's medium |
|---|---|---|---|
| | *Staph. aureus* mg% | *E. coli* mg% | *E. coli* mg% |
| Sulfanilamide | 14.0 | 15.0 | 5.8 |
| Sulfaguanidine | 12.0 | 16.2 | 5.8 |
| Sulfapyridine | 3.0 | 2.9 | .61 |
| Sulfacetimide | 2.0 | 2.8 | .57 |
| Sulfadiazine | .6 | .65 | .061 |
| Sulfathiazole | .3 | .65 | .062 |

salts medium suggested by MacLeod.[2] From a 24-hour culture one million washed cells per ml were inoculated into a flask. The flask was incubated at 37°C for one-half hour to permit adjustment to the conditions of culture before the inoculated medium was dispensed into pyrex tubes containing the drugs. Thus all variations as to strain resistance, size of inoculum, and condition of the medium were eliminated. The tubes were incubated and growth was followed by electrophotometric turbidity measurements.

Table I gives the results of experiments carried out with *Staphylococcus aureus* and *E. coli*. We used as a convenient end-point that concentration of sulfonamide which permitted one-half maximum growth after 16 hours. In this manner the six sulfonamides are divided into pairs according to their effectiveness. This order of potency is not affected by the species of organism or by the medium.

The molecular ratio of the antagonistic pair, *p*-aminobenzoic acid and sulfanilamide, has been the subject of several investigations.[3] It has been pointed out that this ratio is merely a measure of their respective affinity for the unknown receptor site in the organism. If this view is acceptable, then a detailed investigation of these ratios should yield useful information on the general mechanism of sulfonamide action.

Our experiments indicate that these ratios can be used as sensitive and reproducible criteria for the effectiveness of sulfonamides. In such studies one sulfonamide concentration was employed and a range of p-amino benzoic acid concentrations were added to permit estimation of the level restoring growth to the half maximum. Data in support of this procedure are presented in Table II, using one million *E. coli* per ml.

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[2] MacLeod, C. M., *J. Exp. Med.*, 1940, **72**, 217.

[3] Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74; Lockwood, J. S., *Surg. Gynecol. Obstet.*, 1941, **72**, 307; Wyss, O., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 122; Strauss, E., Lowell, F. C., and Finland, M., *J. Clin. Invest.*, 1941, **20**, 189; Spink, W. W., and Jermsta, J., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 395.

TABLE II.
Effect of Concentration of Sulfonamide on Sulfonamide-*p*-Aminobenzoic Acid Ratio.

| Sulfonamide | mg% | P.A.B. mg%* | Molar ratio |
|---|---|---|---|
| Sulfanilamide | 200 | .08 | 2000 |
| ’’ | 100 | .05 | 1600 |
| ’’ | 50 | .02 | 2000 |
| ’’ | 25 | .010 | 2000 |
| Sulfathiazole | 10 | .2 | 27 |
| ’’ | 5 | .1 | 27 |
| ’’ | 1 | .02 | 27 |
| ’’ | .3 | .007 | 23 |

*Concentration required to restore growth to ½ maximum.

The molar ratios of the antagonists are constant for a wide range of concentrations provided that sufficient excess is employed in order that the native sulfonamide inhibitor present in the inoculum does not introduce an appreciable error and provided that the concentration is not so excessive as to affect the physiologic properties of the medium significantly.

The effectiveness of these sulfonamides, determined in terms of their efficiency in overcoming *p*-aminobenzoic acid, is shown in Table III.

From the range of *p*-aminobenzoic acid concentrations employed the level required to restore growth to the half maximum was estimated and the molar ratios of sulfonamide to inhibitor were computed. The similarity of the absolute values (columns 3 and 6) may be fortuitous, but the close agreement of the comparative efficiencies (columns 4 and 7) is highly significant.

A study of this ratio, using two widely differing sulfonamides, was extended to other organisms. *Staphylococcus aureus* and *Proteus vulgaris* were grown on Knight's medium, and *Lactobacillus acidophilus* on a modification of the medium of Snell and Mitchell[4]

TABLE III.
Sulfonamide Efficiency in Knight's Medium.

| Drug | mg% | *Staph. aureus* | | | *E. coli* | | |
|---|---|---|---|---|---|---|---|
| | | PAB mg%* | Molar ratio Drug/PAB | Efficiency | PAB mg%* | Molar ratio Drug/PAB | Efficiency |
| Sulfanilamide | 50 | .0086 | 4660 | 1 | .012 | 3330 | 1 |
| Sulfaguanidine | 50 | .0070 | 4570 | 1 | .0081 | 3960 | .8 |
| Sulfapyridine | 5 | .0066 | 416 | 11 | .0061 | 450 | 7 |
| Sulfacetimide | 5 | .0060 | 534 | 9 | .0060 | 534 | 6 |
| Sulfadiazine | 5 | .030 | 92 | 51 | .064 | 43 | 78 |
| Sulfathiazole | 5 | .050 | 53 | 88 | .065 | 41 | 81 |

*Concentration restoring growth to ½ maximum.

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[4] Snell, E. E., and Mitchell, H. K., *Proc. Nat. Acad. Sci. U.S.*, 1941, **27**, 1.

TABLE IV.
Neutralization of *p*-Aminobenzoic Acid by Sulfanilamide and Sulfathiazole.

| Test organism | Mol. SA/PAB | Mol. ST/PAB | Efficiency ST/SA |
|---|---|---|---|
| *E. coli* | 2,000 | 27 | 74 |
| *A. aerogenes* | 3,220 | 45 | 72 |
| *Staph. aureus* | 4.660 | 53 | 88 |
| *Ps. aeruginosa* | 13,330 | 184 | 73 |
| *Sal. typhimurium* | 6,650 | 92 | 72 |
| *L. acidophilus* | 8,000 | 133 | 60 |
| *Proteus vulgaris* | 4,000 | 55 | 73 |

to which we added a small amount of ether-extracted yeast-extract in place of the purines. The other organisms were grown on MacLeod's medium. The results in Table IV indicate that the efficiency ratio of the two drugs in counteracting the effect of *p*-aminobenzoic acid is strikingly constant.

These data demonstrate the absence of specificity with respect to any of the 7 organisms. A modification of the experiment outlined in Table IV using *E. coli* and sulfanilamide and sulfathiazole, showed that this relation also obtained when the comparative efficiency was determined by plate counts after 2, 4, and 8 hours' growth. Similarly, if peptone was used as inhibitor, the same efficiency ratio held.

Specificity, of course, can be demonstrated under special conditions. For example, as pointed out by Dorfman, *et al.*,[5] an additional inhibition by sulfapyridine is observed when organisms sensitive to nicotinamide deficiency are studied in a medium low in that growth factor. Differences in solubility characteristics, absorption, penetration, maintenance and excretion are also valid practical characteristics of these derivatives which have an important bearing on the clinical aspects of this problem. Our data, however, refer to the fundamental aspects of the action of sulfonamides upon growing bacteria.

This view is a generalization of one aspect of this problem presented by one of us[3] previously. In a wide variety of organisms, the receptor site in the bacterial cell is the same and the difference in effectiveness between the members of this series is a characteristic of the individual sulfonamide and not the species of organism involved.

*Summary.* The molar ratio of *p*-aminobenzoic acid to sulfonamide required to restore a sulfonamide-inhibited inoculum to half maximum growth was determined. Using this ratio as a criterion for the effectiveness of the various sulfonamides, no evidence of specificity was found.

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[5] Dorfman, A., Rice, L., Koser, S. A., and Saunders, F., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 750.

### 13650

# Collodion-Particle Agglutinative Tests in Detection of Antigen Derived from Tubercle Bacilli.*

John T. Riordan. (Introduced by Francis G. Blake.)

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These studies have been devised to explore the range of antigens which react with anti-H37 tubercle-bacillus horse serum and to compare the delicacy of precipitin-tests with collodion-particle agglutination-tests for the detection of these antigens and their corresponding antibodies.

*Materials.* A single lot of anti-H37 tubercle-bacillus serum (designated as No. 5807 A and B) was used in all tests. It was prepared by the Mulford Biological Laboratories of Sharp and Dohme in 1925 by the injection of defatted tubercle bacilli. The immunological properties of this serum have been studied by a number of investigators[1] including Heidelberger,[2] who found its antibody-content to be almost entirely antipolysaccharide. In our own tests the following substances were used as a possible source of antigenic material.

I. *Old Tuberculin* from 4 different companies.

II. *Protein-derivatives of Tuberculin.* (a) P. P. D.—Parke Davis and Company. This purified derivative of tuberculin was dissolved in buffer solution furnished by the company.

(b) T. P. T.—No. 16 (trichloracetic-acid precipitated) prepared at the Henry Phipps Institute of Philadelphia.[†]

III. *Polysaccharides* representing 4 samples derived from various lipids and soluble wax fractions from 3 strains of human tubercle bacilli and one strain of leprosy bacillus. These sugars had been kept in a desiccator since their preparation by Dr. R. J. Anderson.[‡] They were readily soluble in physiological salt solution.

IV. *Crude Aqueous Extracts* from cultures of human tubercle bacilli which had been grown on modified Dorset's and glycerol agar. These extracts were prepared from weighed amounts of

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* Aided by a grant from the John and Mary R. Markle Foundation.

[1] Thomas, R. M., and Duran-Reynals, F., *Yale J. Biol. and Med.*, 1940, **12**, 525.

[2] Heidelberger, M., and Mengel, A. E. O., *J. Biol. Chem.*, 1937, **118**, 79.

[†] I am indebted to Dr. Florence B. Seibert for this preparation.

[‡] I am indebted to Dr. R. J. Anderson of the Department of Chemistry of Yale University for this material.

dried bacilli, suspended in saline solution. The suspensions were then shaken for 20 minutes with glass beads and passed through a Seitz filter. The resulting clear filtrates were used for the tests.

V. *Phosphatide* prepared from human tubercle bacilli by Dr. R. J. Anderson.[‡] Suspensions were made by grinding 10 mg of the phosphatide in 10 cc of distilled water. The resulting suspension was opalescent but showed no tendency to sediment at the end of 48 hours. When diluted $10^{-1}$ it was almost water clear. However, none of the immunological tests proved satisfactory with this colloidal suspension because of the fact that positive reactions were also obtained with normal horse serum.

*Methods.* (a) *Precipitin-reactions* were set up in small tubes, inside diameter 4 mm, using 0.3 cc of antigen and 0.1 cc of anti-H37 tubercle-bacillus serum. They were left in the incubator for 2 hours and then kept in the icebox until read at the end of 24 hours and again at 48 hours. All antigens were set up with normal horse-serum controls.

(b) *Collodion-particle Agglutination.*[§] The principles underlying this technic have been laid down by Freund,[3] and Cannon and Marshall[4] and developed by Goodner.[5] Tests were set up in standard agglutination tubes, with 0.5 cc antigen, 0.1 cc collodion-particle suspension, 0.1 cc antiserum, and 0.3 cc saline. The suspension was added before the antigen and antibody were allowed to combine. The tubes were incubated at 37°C for 2 hours, centrifuged and then read. All tests were set up with normal horse-serum controls.

*Results.* The results of a typical experiment are shown in Table I in which dilutions of antigen have been tested against undiluted serum. As all of the 4 different brands of tuberculin reacted quite similarly, results with only a single brand are shown. This also holds true for the polysaccharides. It will be seen that the collodion-particle agglutinations were about 10 times more delicate than the precipitin-reaction in this series. In Table II antigens have been tested against dilutions of serum. As in Table I no positive readings were encountered with the tuberculoprotein derivatives and so they have not been included. Here, the collodion-particle agglutination is from 8 to 16 times more delicate than the precipitin reaction.

*Summary.* 1. With a single lot of antiserum which has been

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[§] Some of the collodion-particle suspension used in these tests was kindly supplied by Dr. Jules Freund of the New York City Health Department Laboratories.

[3] Freund, J., *Am. Rev. Tuberc.*, 1925, **12**, 124.

[4] Cannon, P. R., and Marshall, C. E., *J. Immunol.*, 1940, **38**, 365.

[5] Goodner, K., *Science*, 1941, **94**, 241.

TABLE I.
Titration of Antigens Against Undiluted Anti-H137 Tubercle-bacillus Serum. Precipitin vs. Collodion-particle Agglutination.

| Dilution of antigen | I<br>Old tuberculin<br>1 cc = 1,000 mg | | IIA<br>P.P.D.<br>1 cc = 0.05 mg | | IIB<br>T.P.T.<br>1 cc = 1 mg | | III<br>Polysaccharide<br>1 cc = 1 mg | | IV<br>Crude aqueous extract of human tubercle bacilli<br>1 cc = 1 mg | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ptn. | C.P.A. | Ptn. | C.P.A. | Ptn. | C.P.A. | Ptn. | C.P.A. | Ptn. | C.P.A. |
| Original | — | 0 | — | — | — | — | ++ | +++ | ++ | +++ |
| $10^{-1}$ | — | +++ | — | — | — | — | ++ | +++ | + | ++ |
| $10^{-2}$ | ++ | +++ | — | — | — | — | + | +++ | — | ++ |
| $10^{-3}$ | ++ | ++ | — | — | — | — | + | ++ | — | + |
| $10^{-4}$ | + | ++ | — | — | — | — | — | + | — | — |
| $10^{-5}$ | — | + | — | — | — | — | — | — | — | — |
| $10^{-6}$ | — | — | — | — | — | — | — | — | — | — |

C.P.A. = Collodion-particle agglutinations:
+++ = disc.
++ = coarse particles.
+ = fine particles.
— = negative.
0 = not done.
Ptn. = Precipitin-reactions:
++ = precipitation in 24 hr.
+ = ” ” 48 ”

TABLE II.
Titration of Antibody (Anti-H37 Tubercle bacillus Serum) Against Various Antigens. Precipitin vs. Collodion-particle Agglutination.

| Dilution of serum | I<br>Old tuberculin<br>1 cc = 10 mg | | III<br>Polysaccharide<br>1 cc = 1 mg | | IV<br>Crude aqueous extract of human tubercle bacilli<br>1 cc = 1 mg | |
|---|---|---|---|---|---|---|
| | Ptn. | C.P.A. | Ptn. | C.P.A. | Ptn. | C.P.A. |
| Original | ++ | +++ | ++ | +++ | ++ | +++ |
| 1:2 | ++ | +++ | ++ | +++ | ++ | +++ |
| 1:4 | + | +++ | + | +++ | + | +++ |
| 1:8 | + | ++ | + | ++ | — | ++ |
| 1:16 | + | ++ | + | ++ | — | ++ |
| 1:32 | — | ++ | — | ++ | — | + |
| 1:64 | — | ++ | — | ++ | — | — |
| 1:128 | — | + | — | + | — | — |
| 1:256 | — | + | — | — | — | — |
| 1:512 | — | — | — | — | — | — |

Legend as in Table I.

artificially produced by injecting defatted tubercle bacilli into horses, a series of comparative immunological tests have been carried out. Results indicate that collodion-particle agglutination-tests are not only satisfactory for this type of immunological determination but are more delicate than precipitin-tests, particularly for the detection of antibody.

### 13651

# Absorption of Crystalline Estrogen from Paraffin Nodules in the Rat.*

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Several methods have been devised to avoid the waste necessarily ensuing from the rapid absorption and excretion of the sex hormones when these substances are injected in oil. Deanesly and Parkes[1] inserted compressed tablets of the crystalline hormones subcutaneously. Noble[2] used a similar method for introducing synthetic estrogens, while Brock and Druckrey[3] placed intraperitoneally collodion sacs containing crystalline estrone. Emmons[4] studied the absorption from compressed tablets inserted subcutaneously, and found minimal variation in the rate for each hormone. With these technics small quantities of hormone were effective for considerably longer periods than is the case when similar amounts are administered in solution.

Geschickter,[5] who reported the occurrence of mammary cancer in the rat approximately 300 days following the subcutaneous implantation of a pellet containing 7.5 mg estrone, stated that it required 4 to 7 months to absorb completely one weighing 3 mg. Mammary lesions resembling neoplasia, which appeared in the rat after a minimum of 226 days, have been described by Noble, McEuen, and Collip[6] as a result of absorption from a compressed tablet of estrone weighing 1 to 7 mg, but the hyperplastic tumor-like lesions regressed following removal of the hormone, or treatment with progesterone.[7]

An effective method for the quantitative estimation of the potency of carcinogenic hydrocarbons has been employed in this Department for a number of years. The carcinogens are dissolved in paraffin wax (melting point 48°C), which is injected subcutaneously in the fluid state. The material solidifies almost immediately as fairly uniform ellipsoids. Statistical analysis of the results support the concept that malignant disease is a local manifestation, a consequence of the direct action of a carcinogen producing a specific cellular injury.[8, 9]

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* Estradiol dipropionate was generously furnished by Dr. R. MacBrayer, Ciba Pharmaceutical Products, Inc., Summit, N.J.

[1] Deanesly, R., and Parkes, A. S., *Proc. Roy. Soc. B.*, 1937, **124**, 279.

[2] Noble, R. L., *The Lancet*, 1938, **2**, 192.

[3] Brock, N., and Druckrey, H., *Klin. Wchnschr.*, 1938, **17**, 23.

[4] Emmons, C. W., *Endocrinology*, 1941, **28**, 633.

[5] Geschickter, C. F., *Radiology*, 1939, **33**, 439.

[6] Noble, R. L., McEuen, C. S., and Collip, J. B., *Canad. M. A. J.*, 1940, **42**, 413.

[7] Noble, R. L., and Collip, J. B., *Canad. M. A. J.*, 1941, **44**, 1.

Hyperplastic mammary alterations were prominent about methylcholanthrene foci in rats and a few mammary carcinomas were produced locally.[8] It was considered probable that only minimal absorption of the hydrocarbons took place, and induced tumors at a distance from the injected material were never observed. No attempt was made to compute the rate of absorption from the nodules, but to judge from the color of those which had lain in animals for long intervals it may be estimated that, regardless of the presence of a tumor, a considerable quantity of the carcinogen was retained locally. That some absorption occurred, however, was evident from the fact that increasing doses of methylcholanthrene caused increasingly high mortality.

*Experimental.* The present communication deals with the absorption and utilization in rats of crystalline estradiol dipropionate incorporated in paraffin. The method has the advantage of simplicity, rapidity, and effectiveness. The estrogen dissolves readily in the proportion of 5 g per 100 cc of paraffin, which is kept liquid in a water bath at 70°C. As 0.1 cc of this solution is administered, the animals receive 5.0 mg crystalline estrogen. Injections are carried out with a tuberculin syringe having a short, large bore needle, likewise kept warm in a test tube immersed in a water bath.

The material was injected subcutaneously in the flanks, thoracic region, or back, intramuscularly in the thigh, or intraperitoneally. Minimal reaction developed about the solidified masses of paraffin, and the ellipsoids eventually became encysted in a thin layer of relatively acellular connective tissue. Some organization occurred in the interior of the nodules. More intense changes suggesting local malignant transformation in the surrounding fibrous tissue have not been observed. In the peritoneal cavity the nodules were generally situated in the greater omentum and here also produced minimal local effects. The location of the nodules exerted no influence on the activity of the hormone, as determined by general influences or on the rate of its utilization.

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[8] Dunning, W. F., Curtis, M. R., and Eisen, M. J., *Am. J. Cancer*, 1940, **40**, 85.

[9] Dunning, W. F., Curtis, M. R., and Wood, F. C., *Am. J. Cancer*, 1940, **39**, 70.

While the results of some investigations[10, 11, 12] appear to indicate that estrogen is inactivated by the liver, the present observations, those of Brock and Druckrey,[3] and of Biskind and Mark,[13] who found that estrone pellets placed in the gastric omentum or the liver were effective, demonstrate that inactivation does not necessarily take place when the total implanted hormone first passes through the liver.

Absorption from the paraffin nodules occurred rapidly, as shown by the production of complete estrus in 48 to 72 hours in infantile rats. Animals 3 to 5 weeks of age of the inbred Sherman strain were employed. With constant absorption of the estrogen during succeeding periods the general effects became readily apparent. The animals were undersized, diffuse mammary hyperplasia was prominent, and ovarian function remained in abeyance, but this was frequently associated with enlargement and squamous cell metaplasia of the uterus, and pyometra. Hyperplasia of the anterior lobe of the pituitary developed relatively late, and was not of the magnitude induced by quickly absorbed oily solutions of the female sex hormone.

Vaginal smears were made weekly. An abrupt disappearance of the estrus smear was assumed to coincide with the complete absorption and utilization of the estrogen. As the object of this experiment was primarily the study of the lesions produced by slowly absorbed estrogen, no additional assays for hormone were conducted on nodules which were no longer effective in animals. In the absence of irreversible damage, as pyometra, other alterations regressed in rats which had utilized completely the estrogen, and normal ovarian function eventually supervened if age did not bar this possibility. Insertion of a new supply of estrogen in paraffin in animals which had reverted to a resting stage again produced the characteristic smear of full estrus. Thus, absorption of a physiologic substance, estrogenic hormone, from a paraffin nodule appeared to proceed more readily than absorption from a nodule containing a carcinogenic hydrocarbon, probably less easily soluble in the body fluids.

The range of variability in the utilization of the 5.0 mg of crystalline hormone is illustrated in the accompanying chart. The earliest negative smear occurred after 48 days, the latest after 301, the mean time for complete utilization being 151 ± 5.9 days.

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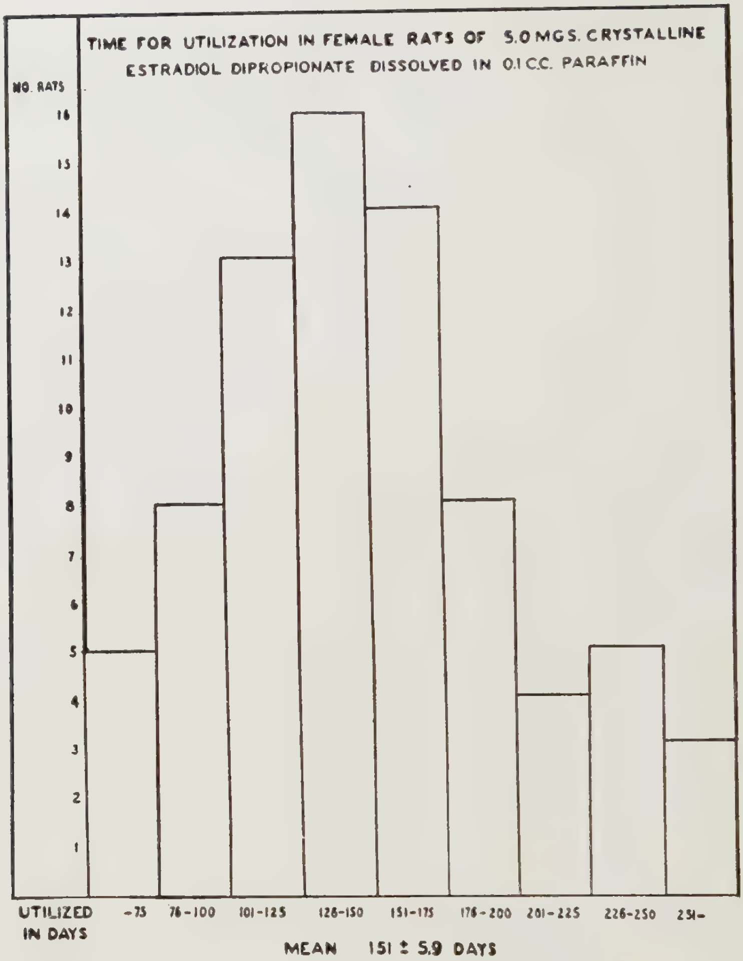
[10] Biskind, G. R., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 266.

[11] Golden, J. B., and Severinghaus, E. L., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 361.

[12] Zondek, B., and Sklow, J., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 276.

[13] Biskind, G. R., and Mark, J., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 212.



In addition to the 76 rats which utilized the estrogen completely, 8 died before the appearance of the first negative smear, while 38 succumbed between 57 and 318 days without utilizing the implanted hormone completely, as was evident from a persisting positive smear.

In 112 males employed, the size and position of the testes and histologic evidence of activity in the seminiferous tubules afforded a comparable indicator of the absorption and utilization of the female sex hormone. The animals used were littermates of the females and received simultaneously similar treatment. The undescended testicles remained rudimentary in size and function. However, after intervals comparable with those observed in females, complete utilization of the hormone became apparent from increasing development of the testes. They now appeared externally, increased gradually to normal size, and eventually descended. Microscopic examination at this time disclosed normally progressing spermatogenesis and normal epithelium in the seminal vesicles. Nevertheless, if males that had utilized completely their first dose of estrogen were given an additional supply in paraffin, the testes gradually decreased in size and reverted to an intraäbdominal position and a rudimentary functional state. Some observations on the temporary character of the testicular atrophy following treatment of males with estrogen have been reported by Deanesly,[14] and on reversible changes in females by Grumbrecht.[15]

*Summary.* An effective and simple method of insuring slow continuous absorption of estrogen in the rat is the incorporation of the crystalline hormone in paraffin (melting point 48°C), which after subcutaneous, intramuscular, or intraperitoneal injection in a liquid state, solidifies locally. As indicated by vaginal smear, the average time required for complete absorption and utilization of 5.0 mg estradiol dipropionate contained in 0.1 cc paraffin, introduced into animals 3 to 5 weeks of age, is 151 ± 5.9 days. In males the position and activity of the testes are comparable indicators of the utilization of the injected estrogenic hormone.

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### 13652

## An Enzyme Which Inactivates Estrone.*

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Zondek[1] was able to demonstrate that liver pulp of homothermal and poikilothermal animals as well as mash of hyacinth roots[2] inactivate estrogenic hormone *in vitro*. Cell-free liver extract yielded similar results. The inactivating factor in liver and hyacinth roots was destroyed by boiling. This led to the conclusion that the inactivation of estrone is due to the action of an enzyme (estrinase).

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[14] Deanesly, R., *J. Endocrinology*, 1939, **1**, 36.

[15] Grumbrecht, P., *Arch. f. Gynäk.*, 1940, **170**, 1.

* This paper has been aided by a grant of the Rockefeller Foundation.

[1] Zondek, B., *Lancet*, 1934, **227**, 356; *Skandin. Arch. Physiol.*, 1934, **70**, 133; *Hormone des Ovariums und des Hypophysenvorderlappens*, 2nd ed., Springer, Vienna, 1935, 124-162; *Genital Functions and Their Hormonal Regulations*, Williams & Wilkins, Baltimore, 1941, 147-175.

[2] v. Euler, H., and Zondek, B., *Biochem. Z.*, 1934, **271**, 64.

Later experiments showed that estrone is also inactivated by potato juice, cauliflower, beetroots and bran[3] as well as by certain bacteria.[4] In every case boiling destroyed the active principle.

The nature of this enzyme is unknown. It is not certain whether the inactivation induced by substances so different as potato juice, liver and bacteria is due to the activity of one or of several different ferments. Heller[5] suggested that 2 different enzymes participate in estrone-inactivation by liver. According to this author an estrone-reducing enzyme contained in liver converts estrone into estradiol and the latter is then transformed by liver dehydrogenase into the inactive substance. Westerfeld[6] assumed, from experiments with mushroom extract, that tyrosinase was the inactivating principle. This was in contradiction to our own observations, in which estrone was found to be inactivated by tyrosinase-free cauliflower juice, yet was unaffected by tyrosinase-containing extracts of mealworm larvae.[3] Graubard and Pincus[7] hold laccase responsible for the inactivation of estrone. We cannot confirm this view, since laccase is present in nearly all plants while estrone-inactivation is produced only by a few plants.[3] In colorimetric determinations the presence of laccase may be masked by vitamin C. For determination of laccase we studied the oxydation of pyrogallol to purpurogallin, after having first destroyed hexuronic acid by oxydation in air at an alkaline reaction. It could thus be shown that juice of apples, tomatoes and onions as well as human saliva contain laccase but fail to inactivate estrone.

It was, therefore, concluded that estrone-inactivation is brought about by a specific enzyme, which is neither tyrosinase nor laccase.

*Technic.* The investigations reported below were concerned with the study of the properties of the estrone-inactivating enzyme contained in potatoes. The concentration of the enzyme in the press juice of potato peel is considerable and therefore such juice lends itself particularly well to the study of the estrone-inactivating factor.

A purified enzyme preparation was obtained in the following manner: Potatoes were thoroughly cleaned with tap water and the peel together with the outer portion of the potato grated on a glass grater. The resulting pulp was pressed through gauze and the juice centrifuged. The sediment, which consisted mainly of starch, was discarded. To the supernatant a saturated solution of ammonium sulphate (SAS) was added until the juice was 0.6 saturated with ammonium sulphate. The precipitate which contained the ferment was then centrifuged and suspended in water to its original volume. The fraction that failed to dissolve was discarded and the solution once more precipitated with SAS as before. The second precipitate was separated by centrifuging, dried in the exsiccator and ground to a fine powder. This material which could be preserved in the exsiccator for a considerable time without loss of potency was used in the following experiments.

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[3] Zondek, B., and Sklow, J., *Endocrinology*, 1942, in press.

[4] Zondek, B., and Sulman, F., *Endocrinology*, 1942, in press.

[5] Heller, C. G., *Endocrinology*, 1940, **26**, 619.

[6] Westerfeld, W. W., *Biochem. J.*, 1940, **34**, 51.

[7] Graubard, M., and Pincus, G., *Proc. Nat. Acad. Sci. U. S.*, 1941, **27**, 149.

Before each experiment 200 mg of powder were shaken for 30 minutes with 5 cc of diluted NaOH (pH 9-9.5) at room temperature (20°). The centrifuged extract was then dialyzed against tap water in a rocking dialyzor for 30 minutes. Owing to loss of alkali the solution became turbid and its pH was between 7.5-7.8. At least 0.02 mg estrone were inactivated by 0.5 cc of the dialyzed solution. The concentration of estrinase in this solution was 4 times higher than in the original potato juice. For the inactivation of 0.02 mg estrone 5.6 mg dry powder of crude potato juice were needed, while an equal effect was produced by only 1.5 mg of dry powder of purified juice. The purified enzyme solution still contained tyrosinase and laccase.

In order to gauge the activity of the purified potato enzyme solution, 0.5 ml was shaken with 1.5 ml phosphate buffer pH 6.4 and 0.02 mg estrone† for 5 hours at 37°. The mixture whose pH was 6.7 was then heated in a boiling water bath and tested for its estrone content on spayed female mice.

To each of the experiments reported below (Nos. 1-7) 40 mice were used, making a total of 280 animals. The test dose was given to 5 groups of 8 animals each, subcutaneously, in 6 injections distributed over a period of 48 hours. Vaginal smears were taken at 12-hour intervals between the 48th and 96th hour after the experiment was begun. The quantity of estrone which produced full cornification (Allen-Doisy Test) was considered to be 1 MU. In our mice, 1 MU is equal to 2 IU. The estrinase unit was defined as that quantity of enzyme which completely inactivates 2 IU = 1 MU of estrone.

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† We are indebted to Dr. Breant, Roche-Organon, Inc., Nutley, N.J., for kindly supplying the estrone. The estrone solution was prepared by adding 1 mg estrone to 0.2 ml of 96 % alcohol and 50 ml n/100 NaOH. The alcohol was then allowed to evaporate in vacuum. The alkaline estrone solution contained 0.02 mg = 200 IU = 100 MU estrone in 1 ml.

*Results.* The following properties of the estrone inactivating enzyme solution of potatoes were noted:

1. The activity is not affected by standing in the open air for 20 hours.
2. All activity is destroyed on heating to 70°C for 45 minutes at pH 7.2. At a more alkaline or more acid reaction destruction takes place even at a lower temperature. At pH 5.5 or 8, heating to 65°C for 45 minutes is sufficient to destroy the active principle.
3. Activity is optimal between pH 6-7 and present in the range between 5-9.
4. In a 2% $H_2O_2$ solution the enzyme is destroyed within 1 hour.
5. In m/500 NaCN solution the enzyme is ineffective.
6. Ultraviolet irradiation of the solution (distance 50 cm) for one hour causes a 20% loss of activity.
7. Dialysis in the rocking dialyzer for one hour does not lessen its potency.

The properties of potato estrinase are thus seen to be closely related to those of tyrosinase, though to some extent different as regards the effect of pH. Tyrosinase is inactive at pH 5, while estrinase retains 50% of its potency at this reaction. The two enzymes are, however, not identical since cauliflower, which contains no tyrosinase, inactivates estrone, whereas the tyrosinase-rich extracts of mealworm larvae do not affect it.

The NCN estrinase reacts similarly to oxidase. A decrease in the estrone-inactivating potency of liver pulp on exposure to m/500 solution of NaCN has also been reported by Heller.[5]

*Summary.* The properties of estrinase, an estrone inactivating enzyme contained in potatoes, are described.

## 13653

# Occurrence of Blood Group Specific Substances in Gastric Juice of Patients with Pernicious Anemia.

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The term "blood group" designates a certain property of the blood. It has been shown that the fixed tissue cells of the entire body exhibit the same group-specific characteristics as the blood cells;[1, 2, 3] and consequently, the label "blood group" does not do justice to the far-reaching group specific differentiation in man. The concentration of group-specific substances in various organs differs considerably. The brain contains very little, if any; the pancreas and intestinal mucosa, on the other hand, are rich in them—more so than the blood cells. Saliva and gastric juice are characterized by a high content of the substances which are secreted continuously as the result of an independent metabolic process.

Roughly 80% of all human beings secrete group-specific substances in both saliva and gastric juice; 20% fail to do so.[4, 5, 6] Secretion and non-secretion are constant and inherited properties and the gene of secretion is dominant over that of non-secretion.[7] The question arises as to whether the capacity to secrete blood-group specific substances is influenced in certain pathological conditions of the gastric mucosa.

Twelve patients with Addisonian pernicious anemia were examined for their capacity to secrete group-specific substances into the gastric juice and saliva. Table I shows the results of the examination of the gastric juice in regard to free and total hydrochloric acid, pepsin content, and pH, as well as the blood group.

Seven of the patients belonged to blood group O, 4 to group A, and one to group AB. The results of the chemical examination of the gastric juices are typical for pernicious anemia.

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[1] Witebsky, E., *Z. f. Immunitätsf.*, 1927, **49**, 517.

[2] Witebsky, E., and Okabe, K., *Z. f. Immunitätsf.*, 1927, **52**, 359.

[3] Kritschewsky, I. L., and Schwarzmann, L. A., *Klin. Wschr.*, 1927, 2081.

[4] Putkonen, T., *Acta Societatis Medicorum Fennicæ "Duodecim,"* 1930, 14.

[5] Lehrs, H., *Z. f. Immunitätsf.*, 1930, **66**, 175.

[6] Friedenreich, V., and Hartmann, G., *Z. f. Immunitätsf.*, 1938, 92.

[7] Schiff, F., Sasaki, H., *Klin. Wschr.*, 1932, **11**, 1426.

TABLE I.
Pernicious Anemia Cases.

| Patient | Blood group | Free HCl | Total HCl cc 0.1 N/100 cc | Pepsin | pH |
|---|---|---|---|---|---|
| 1 | O | X | X | X | 8.65 |
| 2 | O | X | X | X | 7.00 |
| 3 | A | X | X | X | 5.30 |
| 4 | O | none | 7 | none | 6.35 |
| 5 | A | ” | 4 | ” | 6.89 |
| 6 | A | ” | 5 | ” | 6.99 |
| 7 | A | ” | 2 | ” | 6.78 |
| 8 | O | ” | 5 | ” | 6.85 |
| 9 | O | ” | 6 | present | 6.93 |
| 10 | O | ” | 7 | none | 5.88 |
| 11 | O | ” | 8 | ” | 6.98 |
| 12 | AB | ” | 3 | ” | 7.91 |

X—Missing.

The quantitative determination of the group-specific substances in gastric juice and saliva was carried out by means of the so-called inhibition of agglutination test. Table II illustrates an experiment to determine the content of A-specific substance in the gastric juice and saliva of two patients belonging to group A.

Decreasing amounts of saliva and gastric juice respectively (volume 0.2 cc) were kept for 15 minutes at room temperature, together with 0.1 cc inactivated 1:3 diluted human serum belonging to group B. 0.2 cc of a 1% suspension of human red blood cells, group A, were then added to each tube. Agglutination was recorded following centrifugation of the tubes.

TABLE II.
Agglutination of Human Cells Belonging to Group A by Serum of Group B After Treatment of the Latter with Increasing Dilutions of Saliva and Gastric Juice Respectively.

| | Dilutions of gastric juice and saliva | Controls | | Pernicious anemia | | Pernicious anemia | |
|---|---|---|---|---|---|---|---|
| | | a<br>Normal gastric juice<br>Secretor (65)<br>Group A | b<br>Normal Saliva (Wol.)<br>Non-secretor<br>Group A | c<br>Gastric juice | d<br>Saliva | e<br>Gastric juice | f<br>Saliva |
| | | | | Patient Bram.<br>Group A | | Patient Schin.<br>Group A | |
| 1 | Undiluted 0.2 | — | ++++ | — | — | — | — |
| 2 | 1:3 | — | ++++ | — | — | — | — |
| 3 | 1:9 | — | ++++ | — | — | — | — |
| 4 | 1:27 | — | ++++ | — | — | — | — |
| 5 | 1:81 | — | ++++ | — | — | — | — |
| 6 | 1:240 | — | ++++ | — | — | — | — |
| 7 | 1:720 | — | ++++ | — | ± | — | + |
| 8 | 1:2200 | ± | ++++ | — | + | — | + |
| 9 | 1:6600 | + | ++++ | — | +++ | ± | + |
| 10 | 0 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |

— No agglutination.
± Faint agglutination.
+ Slight agglutination.
++ Marked agglutination.
+++ Strong agglutination.
++++ Very strong agglutination.

It may be seen from Table II that gastric juice and saliva from 2 patients suffering from pernicious anemia contain the A substance. The amount secreted in the gastric juice seems to be at least as large as that of normal gastric juice. The examination of the 4 cases belonging to group A, as well as of the AB case, gave essentially identical results in regard to the secretion of A specific substance. All 5 cases were secretors. The AB case secreted both the A and the B substance into the gastric juice as proved by the inhibition of agglutination test. No case belonging to group B was among the group of the 12 pernicious anemia cases which we had the opportunity to examine.

Inasmuch as 7 of the 12 cases of pernicious anemia belonged to group O, it seemed desirable to determine whether the O substance was secreted in the gastric juice. The demonstration of the O substance is possible by means of certain beef sera. The technic used in the preparation of anti-O beef sera has been referred to in a previous communication.[8] Briefly, certain selected beef sera, after being absorbed with red blood cells belonging to group $A_1B$ agglutinate red blood cells of group O and of group $A_2$, while they agglutinate $A_1$ cells and B cells only slightly.

A typical experiment carried out in order to show the presence or absence of the O specific substance in the saliva and gastric juice of 2 patients belonging to group O is recorded in Table III. Decreasing amounts of gastric juice (volume 0.2 cc) were mixed with 0.1 cc of 1 :4 diluted anti-O beef serum No. 11. The tubes were kept at room temperature for 15 minutes and then 0.1 cc of 1% suspension of

TABLE III.

Agglutination of Human Cells of Group O by Anti-O Beef Serum After Treatment of the Latter with Increasing Dilutions of Saliva and Gastric Juice Respectively.

| | Control | Pernicious anemia | | Pernicious anemia | |
|---|---|---|---|---|---|
| | a | b | c | d | e |
| Dilutions of gastric juice and saliva | Normal gastric juice Non-secretor Group O | Gastric juice | Saliva | Gastric juice | Saliva |
| | | Patient Dun. Group O | | Patient Durk. Group O | |
| 1 Undiluted 0.2 cc | ++ | + | +++ | — | — |
| 2 1:3 ” ” | ++ | + | +++ | — | — |
| 3 1:9 ” ” | +++ | ++ | +++ | — | — |
| 4 1:27 ” ” | +++ | ++ | +++ | — | ± |
| 5 1:81 ” ” | +++ | +++ | +++ | ± | + |
| 6 1:240 ” ” | +++ | +++ | +++ | + | + |
| 7 1:720 ” ” | +++ | +++ | +++ | + | + |
| 8 1:2200 ” ” | +++ | +++ | +++ | ++ | + |
| 9 0 ” ” | +++ | +++ | +++ | +++ | +++ |

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[8] Witebsky, E., and Klendshoj, N. C., *J. Exp. Med.*, 1941, **73**, 655.

human blood cells belonging to group O were added. The tubes were kept again for 15 minutes at room temperature and centrifuged.

Table III shows that the agglutination of human group O cells by anti-O beef sera is inhibited by the gastric juice and saliva of patient Durk., proving the presence of O substance in both. Patient Dun. apparently did not secrete the O substance in the gastric juice, since the agglutination of O cells is inhibited only slightly by the gastric juice of this patient. The examination of his saliva, also, showed a complete absence of O specific substance, indicating that this patient belongs to the group of non-secretors.

*Conclusion.* The gastric juice of patients suffering from pernicious anemia contained blood group specific substances at least in the same large amounts as normal gastric juice in spite of the fact that there was no free hydrochloric acid or pepsin present in the specimens. Eleven of the 12 patients were proved to belong to the so-called secretor group, while one was a non-secretor.

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**13654**

# I. Nature of Urobilin Obtained after Amalgam Reduction of Human Fistula Bile.*

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The reduction of crystalline bilirubin either by amalgam or catalytic methods is known to yield the well-defined chromogen, mesobilirubinogen.[1-4] The *in vitro* oxidation of mesobilirubinogen leads to urobilin IX,a,[†] not to stercobilin.[5, 6, 7] The latter substance as

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[*] Aided by a grant from the John and Mary R. Markle Foundation, New York City.

[1] Fischer, H., *Z. physiol. Chem.*, 1911, **73**, 204.

[2] Fischer, H., and Meyer-Betz, F., *Z. physiol. Chem.*, 1911, **75**, 232.

[3] Fischer, H., and Hess, R., *Z. physiol. Chem.*, 1931, **194**, 193.

[4] Fischer, H., and Adler, E., *Z. physiol. Chem.*, 1931, **200**, 209.

[†] This substance was first described under the name of K-urobilin.[6] The designation IX,a simply indicates a configuration corresponding with mesoporphyrin IX, the porphyrin ring being opened at the a-methene bridge.

[5] Watson, C. J., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1508.

[6] Watson, C. J., *Z. physiol. Chem.*, 1935, **233**, 39.

[7] Siedel, W., and Meier, E., *Z. physiol. Chem.*, 1936, **242**, 101.

isolated in crystalline form from human feces and "urobilin"-containing urines, is optically active, strongly laevorotatory, while urobilin IX,a is optically inactive.[8, 9] The reduction of stercobilin yields stercobilinogen and not mesobilirubinogen.[5, 6, 9, 10]

In considering the *in vivo* transition of bilirubin to stercobilin, the question arose as to whether 2 bilirubins might be present in the bile, one the mother substance of mesobilirubinogen and urobilin IX,a, the other of stercobilinogen and stercobilin. Fischer and Libowitzky[11] recently isolated bilirubin from human bile. Reduction of this bilirubin led only to mesobilirubinogen. This finding, however, did not exclude the possibility that the bilirubin in its natural state in the bile was in some way modified so that if reduction were carried out on the bile directly, stercobilinogen might be formed.

In the present investigation, 7 samples of human fistula bile were used. Samples 1-4 did not contain native urobilin or urobilinogen, and were therefore subjected at once to amalgam reduction. This consisted in shaking the diluted bile with an excess (50-200 g) of 3.5-4% sodium amalgam, until nearly colorless. The Ehrlich reaction had now become strong. The samples were acidified weakly with glacial acetic acid and were then extracted repeatedly with petroleum ether. Samples 5-7 contained native urobilinogen and were therefore acidified and extracted with petroleum ether prior to the amalgam reduction. The latter was then carried out on the aqueous fraction and after acidification the newly formed urobilinogen was extracted with petroleum ether. The term urobilinogen is used here to indicate the sum of any mesobilirubinogen, and stercobilinogen, or other Ehrlich reacting chromogen that might have been formed. The petroleum ether extracts before amalgam reduction, and containing the native urobilinogen are designated in the following as 5-7 a, while those of the bile after reduction are 5-7 b.

The petroleum ether extracts in each instance were further treated as follows: Filtered through petroleum ether-treated paper—allowed to stand in the light for several days until the Ehrlich reaction was weak or negative—precipitated urobilin collected and redissolved in a small amount of $CHCl_3$—urobilin reprecipitated by pouring into 6 volumes of petroleum ether—dry precipitate dissolved in 1% $Na_2CO_3$—washed with ether, then acidified with acetic acid—washed with ether, then made 7% HCl by addition of sufficient

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[8] Fischer, H., Halbach, H., and Stern, A., *Ann. d. Chem.*, 1935, **519**, 254.

[9] Fischer, H., and Halbach, H., *Z. physiol. Chem.*, 1936, **238**, 59.

[10] Watson, C. J., *Z. physiol. Chem.*, 1932, **208**, 101.

[11] Fischer, H., and Libowitzky, H., *Z. physiol. Chem.*, 1939, **258**, 255.

25% HCl—urobilin hydrochloride removed by repeated extraction with small amounts of $CHCl_3$—dried and concentrated to a small volume *in vacuo*. When crystallization could not be achieved directly by cooling and scratching it was brought about by concentrating further to dryness and washing the residue with cold acetone.[7] This permitted separation of dark impurities from the light orange urobilin which was then easy to crystallize from hot acetone or from $CHCl_3$-acetone. Crystals were obtained from samples 1, 2, 5b, 6b, and 7b. The additional step of washing the initial $CHCl_3$ residue with cold acetone would probably have permitted crystallization from samples 3 and 4 as well, but at the time these were studied, the value of the procedure was not recognized. In each instance the crystals were of the boat-shaped type characterizing urobilin IX,a from acetone.[7] The melting points[‡] were likewise indicative of this substance rather than of stercobilin. This may be seen in Table I.

Optical activity was determined for each sample, either for the crystals dissolved in $CHCl_3$ or for the final concentrated $CHCl_3$ solution from which crystallization had not occurred. For the sake of control it was repeatedly determined that there was measurable laevo-rotation in less concentrated solutions of crystalline stercobilin (isolated from the feces). Our measurements for stercobilin agree with those of Fischer and coworkers,[8, 9] $[\alpha]_{589}^{20} = -3300°$ ($CHCl_3$). Relative concentration was determined by means of absorption intensity as measured with the Evelyn photo-electric colorimeter, filter 500. Determinations of optical activity were made with a Schmidt-Haensch polarimeter, employing a sodium lab-arc as light source. In each instance the urobilin obtained after the amalgam reduction of the bile, was optically inactive (samples Nos. 1, 2, 3, 4, 5b, 6b, and 7b). Strong dextrorotatory activity was en-

TABLE I.
Melting Points of Urobilin IX,a, Stercobilin, and Crystals Isolated from Present Samples.

| | | | Present samples | | | |
|---|---|---|---|---|---|---|
| | Urobilin IX,a | Stercobilin | 1 | 2 | 6b | 7b |
| Hydrochloride (from $CHCl_3$) | 145-150°C | 125-128°C | | 145-152°C | | |
| Hydrochloride (from acetone) | 165-170°C | 146-149°C | | | 167-170°C | 165-168°C |
| Free substance (from acetone) | 176°C (7) | 236°C (9) | 140-150°C (one crystallization only) | | 174-176°C (recryst.) | |

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[‡] With exception of sample No. 1, in which an ordinary macro-method was used, the melting points were determined with a Fisher-Johns micro-apparatus. The melting point of the crystals from sample 5b was not determined.

TABLE II.

| Stercobilin | Urobilin IX,a | Crystals, 6b |
|---|---|---|
| 4922 Å | 4952 Å | 4948 Å |
| 4921 | 4951 | 4952 |
| 4925 | 4950 | 4948 |
| 4922 | 4947 | 4952 |
| Avg 4923 | Avg 4950 | Avg 4950 |

Solvent: 5 cc methyl alcohol + 2 drops 10% HCl.

countered in the native urobilins present in the final $CHCl_3$ solutions from samples 5a and 7a. The presence of a dextrorotatory urobilin in infected fistula bile will be described in a separate communication.

The spectroscopic studies confirmed Lemberg's observation[12] that a slight difference exists in the position of the visible absorption band of stercobilin and urobilin IX,a. Table II contains a series of values for crystalline stercobilin (from feces), crystalline urobilin IX,a (from mesobilirubinogen), and the crystals obtained from sample 6b.

A difference of the same magnitude, *i. e.*, 25 Å, was observed both by measurement and superimposition of absorption spectra with the solutions of the crystals obtained from samples 1, 2, 5b, and 7b.

*Discussion.* The present results reveal clearly that simple reduction of the bilirubin present in human bile does not lead to the formation of stercobilinogen and hence to stercobilin. The lability of the urobilin which is obtained, together with its manner of crystallization, melting point, absorption spectrum, and total lack of optical activity are regarded as proof of identity with urobilin IX,a. Thus there is no reason to assume the presence of a second or modified bilirubin in the bile. This would have been a prominent possibility if stercobilin had appeared following these *in vitro* reductions. The present results also constitute further evidence that in the process of formation of bile pigment the protoporphyrin ring in the hemoglobin molecule is opened only at the a-methene bridge. Without this evidence the occurrence of differing tetrapyrrylmethenes, either bilirubins or bilirubinoid in character, could not be excluded, since it was conceivable, *a priori*, that the porphyrin ring might open at one of the other methene bridges. The present results, in agreement with those of Fischer and Libowitzky,[11] indicate that this is very unlikely.

Since there can be no doubt that stercobilin is a bilirubin derivative,[9] it becomes evident that it must owe its formation to a further chemical activity peculiar to the lumen of the intestinal tract. Thus we cannot agree with the suggestion of Lemberg and coworkers[12] that the term stercobilin be discarded, since in view of the present results it appears to be most descriptive insofar as stercoraceous mode and site of origin are concerned. Further studies of the transition of bilirubin to stercobilin will be described in separate communications of this series.

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[12] Lemberg, R., Lockwood, W. H., and Wyndham, R. A., *Austral. J. Exp. Biol. and Med. Sc.*, 1938, **16**, 169.

Much confusion has arisen with respect to the terminology of the natural bilirubin derivatives. In earlier papers it was stated[5, 6, 13, 14] that stercobilin was identical with natural urobilin as isolated from the urine. This was misinterpreted[7] to mean that stercobilin was identical with urobilin (IX,a) as derived from mesobilirubinogen, although the distinction of these substances had been demonstrated repeatedly.[5, 6, 15] The point of confusion, of course, was the term urobilin. We believe that confusion will be avoided in the future if the term urobilin IX,a is used to designate the optically inactive urobilin formed by simple dehydrogenation of mesobilirubinogen. The term stercobilin should apply only to the laevorotatory type of urobilin as originally isolated from feces.[10, 16]

*Summary and Conclusions.* The amalgam reduction of the native bilirubin in 7 samples of human fistula bile led only to mesobilirubinogen which in turn yielded urobilin IX,a, on suitable oxidation. No evidence of stercobilin formation was obtained. The formation of stercobilin is thus shown to depend upon a further chemical activity which is peculiar to the intestinal tract.

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[13] Watson, C. J., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 1210.

[14] Watson, C. J., *Z. physiol. Chem.*, 1933, **221**, 145.

[15] Watson, C. J., *J. Biol. Chem.*, 1936, **114**, 47.

[16] Watson, C. J., *Z. physiol. Chem.*, 1932, **204**, 57.

### 13655 P

## II. Isolation of a Dextrorotatory Urobilin from Human Fistula Bile.*

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The formation of urobilinogen or urobilin in infected bile has been noted repeatedly in the past.[1, 2] MacMunn[3] observed that the urobilin in the bile of humans, mice, cattle, hogs and sheep differed slightly in spectroscopic characteristics from urinary urobilin. There has been no subsequent investigation of this possible difference.

We have repeatedly observed that fistula bile becomes much lighter in color on standing at room temperature or in the incubator. Coincident with this lightening, the Ehrlich reaction for urobilinogen becomes much more intense, in some instances having been negative at the outset. These changes are associated with an obvious and marked increase in bacterial flora and with the appearance of a fecal odor. The urobilinogen which forms during this period is readily oxidized to a urobilin, which is strongly dextrorotatory, quite unlike stercobilin (laevorotatory), or urobilin IX,a (optically inactive). This dextrorotatory urobilin has been identified in 11 of 12 bile samples from 9 different patients. Since some of these samples exhibited a strong Ehrlich reaction as they left the fistula tube, it is likely that the formation of the dextrorotatory urobilinogen was proceeding in the infected biliary tract. The possibility exists, however, that the chromogen first excreted was mesobilirubinogen, since as will be seen in the next paper of this series, mesobilirubinogen is converted to d-urobilin in some samples of bile. The substance has been isolated in crystalline form from four of these samples. The method of isolation may be given briefly as follows: The bile is weakly acidified with 10% HCl, then extracted 5 times with $CHCl_3$. The latter is filtered through a $CHCl_3$ moistened paper and is then passed through a Tswett column of $Al_2O_3$† 8 x 2 cm. The urobilinogen and urobilin are adsorbed near the top of the column while

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* Aided by a grant from the John and Mary R. Markle Foundation, New York City.

[1] Meyer-Betz, F., *Ergeb. d. inn. Med. u. Kinderheilk.*, 1913, **12**, 733.

[2] McMaster, P. D., and Elman, R., *Ann. Int. Med.*, 1927, **1**, 88.

[3] MacMunn, C. A., *Proc. Roy. Soc.*, 1881, **31**, 206.

† According to Brockmann; Merck & Company.

bilirubin wanders down slowly. The column is next washed with ethyl alcohol (30-50 cc) to remove $CHCl_3$. The urobilinogen and urobilin are then eluted completely with a 20% aqueous solution of acetic acid.[‡] The acetic acid eluate is allowed to stand over night to permit oxidation of urobilinogen to urobilin. The further treatment is as follows: The eluate is washed repeatedly with ether, acidified further by adding a 1/5 volume of concentrated HCl, following which the urobilin hydrochloride is extracted with $CHCl_3$. The latter is filtered through $CHCl_3$ moistened paper and concentrated to dryness on the boiling water bath. Acetone is added to the hot residue with resultant immediate crystallization. The intense green fluorescence with alcoholic zinc acetate is the same as for stercobilin and urobilin IX,a. The absorption band in methyl alcohol containing a little HCl is identical with that of urobilin IX,a; *i. e.*, at a maximum of 4945 Å, while the stercobilin maximum is at 4920 (see Paper I). This undoubtedly explains the difference noted by MacMunn, since stercobilin predominates in feces and in many pathological urines. The specific rotatory activity is in the neighborhood of $[\propto]_{20^\circ}^{589\,m\mu} = +4000^\circ$. As yet the yields of this substance have not exceeded 1-2 mg, so that this value cannot be regarded as more than approximate. Stercobilin has been shown to be dextrorotatory in concentrated sulphuric acid and in formic acid.[4] We cannot relate these observations to the present d-urobilin, however, since in the case of sulphuric acid the laevorotatory activity is restored when the substance is taken into $CHCl_3$, and in the case of formic acid the dextrorotatory activity is undoubtedly due to salt formation,[4] while we have ascertained that the free d-urobilin of itself exhibits strong dextrorotation. The melting point of the hydrochloride is approximately that of stercobilin; *i. e.*, 142-144°C (from acetone), 128-131°C (from $CHCl_3$).[§] The melting point of the free substance has not yet been ascertained.

A specific color reaction with dioxan-HCl has been noted and is receiving further study. When a solution of d-urobilin in dioxan + 1-2 drops of 10% HCl is heated in a boiling water bath for 2-5 minutes, the color changes from orange, to violet, to blue, to green. Stercobilin is unaffected by the same treatment; urobilin IX,a becomes violet after 5-10 minutes and soon becomes colorless. Bilirubin exhibits a transitory green color with this method.

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[‡] Bilirubin is not eluted by 20% acetic, but is removed subsequently by $CHCl_3$ containing 1% glacial acetic, from which mixture it crystallizes readily. This method of isolation of bilirubin from bile will be described in detail in a separate communication.

[4] Fischer, H., and Libowitzky, H., *Z. physiol. Chem.*, 1939, **258**, 255.

[§] These melting points were determined with a Fisher-Johns micro-apparatus.

It is believed best to designate this new substance tentatively as d-urobilin, at least until further evidence regarding its structure is obtained. This designation does not necessarily imply that d-urobilin is a stereo-isomer of stercobilin, although such a relationship is suggested by the identical melting points of the hydrochlorides of the two substances. Further studies including elementary analyses are in progress.

*Summary and Conclusions.* 1. A dextrorotatory urobilin has been identified in 11 of 12 infected fistula bile samples examined. 2. The initial urobilinogen content of infected bile samples is considerably increased and the bile becomes lighter in association with overgrowth of bacteria productive of a fecal odor. The crystalline urobilin isolated from four of such samples was dextro- rather than laevorotatory (as in the case of stercobilin isolated from the feces). 3. A specific color reaction and other characteristics of d-urobilin are described.

## 13656

## III. Formation of D-Urobilin from Mesobilirubinogen in Human Bile.*

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The presence of a dextrorotatory urobilin in infected bile samples, as described in the preceding paper of this series, induced us to study the question of derivation of this substance. Since the simple reduction of bilirubin, *in vitro,* leads only to mesobilirubinogen, the first possibility requiring investigation was that d-urobilin was derived from mesobilirubinogen through some chemical activity peculiar to the bile. The following experiments were carried out with a view to answering this question.

1. *Addition of Mesobilirubinogen to Urobilin-free Bile.* Fifty mg of crystalline mesobilirubinogen were dissolved in 1 cc 95% ethyl alcohol and 5 cc of 0.025% NaOH. This mixture was added

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* Aided by a grant from the John and Mary R. Markle Foundation, New York City.

to 10 cc of urobilin and urobilinogen-free fistula bile, which did not have a fecal odor. After thorough mixing, the bile and mesobilirubinogen in a small glass container were placed in the upper portion of an ordinary vacuum jar. The glass container rested on a wire gauze above the lower compartment which was partially filled with water. As a control, 10 cc of the same bile without added mesobilirubinogen but with 1 cc of 95% alcohol and 5 cc of 0.025% NaOH were similarly placed in the jar. The jar was then evacuated to a point where the bile commenced to rise, after which it was placed in an incubator at 37°C. Five days later the samples were removed and subjected to the following treatment: 100 cc of 7% HCl were added to each bile sample after which it was allowed to stand over night at room temperature. The Ehrlich reaction, which had been intense, was now weak. The diluted HCl-bile was now filtered and extracted repeatedly with $CHCl_3$. The latter was filtered through $CHCl_3$-treated paper to remove moisture after which it was concentrated to dryness on the boiling water bath. Acetone was at once added and crystallization then occurred in each instance. In the first, to which mesobilirubinogen had been added, the crystals were those of a urobilin together with a small amount of crystalline bilirubin. A solution of these crystals in 60 cc of $CHCl_3$ exhibited +0.13° (corr.[†]) optical activity with sodium light. The limit of error with the Schmidt-Haensch polarimeter used was ± 0.015°. The solution exhibited strong urobilin absorption and green fluorescence with alcoholic zinc acetate. The crystals obtained from the control, on the contrary, appeared to be solely bilirubin, exhibiting neither optical activity, urobilin absorption, nor green fluorescence with zinc.

2. *Addition of Mesobilirubinogen to Urobilin Containing Bile.* In a similar experiment 58 mg of mesobilirubinogen were added to 10 cc of infected bile with fecal odor, containing 5.5 mg of native urobilinogen. This was placed in a partly evacuated jar in the incubator, as described above. 150 cc of the same bile were placed in the refrigerator, to be used as a control. The latter was studied further after 3 days and the 10 cc incubated sample after 12 days. Both were subjected to the chromatographic method described in Paper II. The urobilin crystals obtained from each sample were dissolved in 15 cc of $CHCl_3$. The solution from the control (150 cc) sample exhibited +0.30° rotation, while that from the sample (10 cc) to which mesobilirubinogen had been added had +0.21°

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[†] The actual readings with this apparatus are 3 times greater than the corrected values for degrees of optical activity.

rotation. If no mesobilirubinogen had been added, the expected rotation on the basis of the control would have been only +0.02°. The objection might be raised that the tenfold increase which was observed was due to conversion of bilirubin to d-urobilin. It is believed, however, that the amount of bilirubin in the 10 cc sample was much too small to be of significance. To account for the difference the sample would have had to contain 55 mg of bilirubin, and all of this would have had to be reduced, which was obviously not the case since crystalline bilirubin was obtained following chromatographic analysis.

3. *The Effect of Acholic Feces on Urobilin-free Bile and on Mesobilirubinogen.* (a) Control sample. A 10 g sample of feces containing 1.4 mg urobilinogen by quantitative method.[1] While not absolutely acholic, this represents a high degree of exclusion of bile from the intestinal tract. This was incubated in an evacuated jar for 6 days, as described above. Ground and extracted with 7% HCl. Filtered. Filtrate extracted with $CHCl_3$. Filtered through $CHCl_3$-moistened paper and concentrated to dryness. Acetone added. Amount too small to crystallize. Optically inactive.

(b) 10 g of same feces as in (a) + 46 mg crystalline mesobilirubinogen. Thoroughly mixed. Incubated and further treated as in (a). Crystalline urobilin IX,a isolated. Optically inactive.

(c) 10 g of same feces as in (a) + 50 mg mesobilirubinogen + 5 cc urobilin-free bile. Mixed, incubated, and further treated as in (a). Crystalline d-urobilin obtained. Solution of crystals in 15 cc $CHCl_3$ exhibited (+) 0.07° rotation (corr.).

(d) 20 cc urobilin-free bile + 1 g of the same feces as in (a). Mixed, incubated, and further treated as in (a). Crystalline d-urobilin obtained. Crystals in 20 cc $CHCl_3$ exhibited optical activity of (+) 0.13° (corr.).

4. *The Effect of Bile on Stercobilin.* The question arose as to whether stercobilin or stercobilinogen was converted in the bile to d-urobilin. This has not yet been answered with certainty, but the following observations indicate that it is not.

(a) Crystalline stercobilin was isolated[‡] on one occasion from a sample of bile from a patient with incomplete bile fistula (T-tube without suction). The method used was essentially the same as the chromatographic procedure described in Paper II of this series. The observed laevorotation of the crystals in $CHCl_3$ was (–)0.10° (corr.).

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[1] Watson, C. J., *Am. J. Clin. Path.*, 1936, **6**, 458.

[‡] We are indebted to Dr. Edmund Flink for this isolation.

(b) 50 mg of stercobilin hydrochloride were injected intravenously[2] in a patient having a complete (suction) external biliary fistula.[3] The bile obtained just before the injection gave negative Ehrlich and Schlesinger tests for urobilinogen and urobilin respectively. The sample collected during the first hour after injection was strongly positive for both substances. During the ensuing 11 hours the reactions became gradually weaker and were negative thereafter. By employing the chromatographic procedure described in the preceding paper, it was possible to obtain crystalline stercobilin from the combined bile samples for the 12-hour period after the injection. In $CHCl_3$ an optical activity of (–)0.045° was observed.

*Discussion.* The present results indicate that mesobilirubinogen is converted to d-urobilin in bile whether the latter is initially urobilin-free or urobilin-containing. In the preceding paper it was noted that the formation of urobilinogen in bile standing at room temperature or in the incubator is associated with the growth of bacteria productive of a fecal odor. This type of activity is evidently necessary for the reduction of bilirubin to a urobilinogen (either mesobilirubinogen or d-urobilinogen). It is just as evident, however, that if mesobilirubinogen is added to bile it may be converted to d-urobilin without this type of bacterial activity. Studies are now in progress to determine, if possible, the relation of bacteria to this problem, and also to the problem of stercobilin formation from mesobilirubinogen, as discussed in Paper IV of this group.

It is important to note that in the present study and in additional experiments that will be described subsequently, acholic feces alone did not convert mesobilirubinogen either to stercobilin or d-urobilin. Acholic feces + bile in 2 instances brought about conversion of mesobilirubinogen to d-urobilin. This was significant inasmuch as the control sample on incubation failed to develop urobilinogen or urobilin. As will be noted in Paper IV, the conversion is not uniformly in this direction and it appears that the direction with any given mixture of feces and bile depends upon a combination of factors as yet unknown. It will be shown, however, that with normal feces, or in the normal intestinal tract, mesobilirubinogen is converted to stercobilin rather than d-urobilin.

*Summary and Conclusions.* 1. Mesobilirubinogen added to both urobilin-free and urobilin-containing bile was converted at least in part to d-urobilin. No evidence of stercobilin formation was obtained. 2. Mesobilirubinogen added to bile and acholic feces was converted at least in part to d-urobilin. In one instance the addition of acholic feces to urobilin-free bile was instrumental in the development of d-urobilin.

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[2] Watson, C. J., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 377.

[3] Layne, J. A., and Bergh, G. S., *Surgery*, 1941, **10**, 563.

### 13657

## IV. Formation of (Laevorotatory) Stercobilin from Mesobilirubinogen in Human Feces.*

C. J. Watson, Victor Sborov and Samuel Schwartz.

*From the Division of Internal Medicine, University of Minnesota Hospital, Minneapolis, Minn.*

In Paper I of the present series it was shown that stercobilin could not be obtained by the amalgam reduction of native bilirubin in various bile samples. This led only to mesobilirubinogen and, on oxidation, urobilin IX,a. Nor was stercobilin obtained by incubating mesobilirubinogen with acholic feces, or with bile, as described in Paper III. In these experiments, the hitherto undescribed d-urobilin was formed.

In the present study the following experiments were carried out in order to determine whether mesobilirubinogen is converted to stercobilinogen in the intestinal tract, or when incubated *in vitro* with normal feces.

1. 210 mg of crystalline mesobilirubinogen were dissolved in 50 cc of dilute $Na_2CO_3$ (0.2%) to which was added 0.33 g bile salts (Bilien, Abbott). The solution was diluted further to 100 cc with physiological saline. This was administered over a period of one-half hour through a Miller-Abbott tube, into the duodenum of a patient having a T-tube in the common bile duct, and with a continuous suction fistula.[1] The urobilinogen excretion in the feces was determined for two-day periods before and after the administration of mesobilirubinogen as shown in Table I. The feces for the various periods indicated by the brackets on the left in Table I were subjected to the chromatographic isolation method as described in Paper II of this series, with the one exception that the primary extraction and adsorption was carried out with 95% ethyl alcohol rather than with $CHCl_3$. The relative advantages of various isola-

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* Aided by a grant from Mr. A. C. Randall, Minneapolis.

[1] Layne, J. A., and Bergh, G. S., *Surgery*, 1941, **10**, 563.

TABLE I.

| Date | Feces urobilinogen in mg per day | Crystals obtained | Degrees rotation in 15 cc $CHCl_3$ | Comment |
|---|---|---|---|---|
| 3/6 -3/8 | 11.6 | — | (–)0.11° | Continuous suction on 3/2-3/18 |
| 3/10-3/12 | 7.0 | | | |
| 3/12-3/14 | 19.8 | + | (–)1.02° | 210 mg mesobilirubinogen on 3/13 |
| 3/14-3/16 | 47.3 | | | |
| 3/16-3/18 | 11.4 | Isolation not attempted | | |
| 3/18-3/21 | 55.8 | + | (–)0.97° | Bile suction discontinued |

tion procedures for urobilin and stercobilin will be discussed in a separate communication. It is seen in Table I that stercobilin increased markedly in the feces after the administration of mesobilirubinogen. A comparison of the total urobilinogen and optical activity values for this period with those of 3/18 to 3/21 reveal striking agreement. In the latter period the only source of urobilinogen was the native bilirubin of the patient's bile.

2. Feeding of mesobilirubinogen. Subject A.R.,[‡] ♂, 73. 775 mg of crystalline mesobilirubinogen were fed, in capsules, over a period of 12 hours. The feces urobilinogen was determined before, during, and after the time of administration. The various samples were then subjected to alcohol extraction and chromatographic separation as already referred to. The data are shown in Table II.

A significant increase in stercobilin is observed in Table II during the period following administration of the mesobilirubinogen.

3. Incubation of mesobilirubinogen with feces. The methods employed in these experiments were entirely similar to those described above and in the preceding papers of this series. In Table III are shown the data from four experiments in which mesobilirubinogen was added either to acholic or normal feces. The fourth of these experiments was already described in the preceding paper and the

TABLE II.

| Date | Feces urobilinogen in mg per day | Mg stercobilin* | | |
|---|---|---|---|---|
| | | Crystals | Mother liquor | Total |
| 6/19-6/23 | 197.5 | 11.5 | 38 | 49.5 |
| 6/23-6/27 | 243.8 | 19 | 21 | 40 |
| 6/27 | 775 mg mesobilirubinogen *per os.* | | | |
| 6/27-6/29 | 235 | 49 | 44 | 93 |
| 6/29-7/1 | 308 | | | |
| 7/1 -7/5 | 165 | 29 | 5 | 34 |

*In terms of optical activity. Calculated from $[\alpha]_{20^\circ}^{589} = -3300^\circ$ ($CHCl_3$).

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[‡] This patient had a mild refractory anemia of undetermined origin.

TABLE III.

| Grams feces | Total mg urobilinogen in feces | Material added | Incubation days | Method of purification | Stercobilin in mg* | | |
|---|---|---|---|---|---|---|---|
| | | | | | Crystals in $CHCl_3$ | Mother liquor | Total mg |
| 1. | | | | | | | |
| a. 20 | 33.5 | — | 3 | Chromatographic | <1 | 1.8 | 2+ |
| b. 20 | 33.5 | 58 mg mesobilirubinogen | 3 | ” | 5 | 9.5 | 14.5 |
| 2. | | | | | | | |
| a. 20 | 0 | 64 mg ” | 3 | ” | 0† | 0 | 0 |
| b. 20 | 0 | 69 mg ” | 0 | ” | 0† | 0 | 0 |
| 3. | | | | | | | |
| a. 10 | 0 | 100 mg ” | 5 | 7% HCl-$CHCl_3$ | 0† | 0 | 0 |
| b. 10 | 0 | 100 mg ” <br> + 5 cc bile‡ | 5 | 7% ” ” | 0.8 | not determined | >1 |
| 4. | | | | | | | |
| a. 10 | 1.4 | — | 6 | 7% ” ” | 0 | 0 | 0 |
| b. 10 | 1.4 | 46 mg mesobilirubinogen | 6 | 7% ” ” | 0† | 0 | 0 |

*Calculated on basis of optical activity $[\alpha]_{20°}^{589} = (-)\ 3300°$ ($CHCl_3$).

†Crystals of urobilin IX,a, isolated (optically inactive).

‡Weakly positive Ehrlich reaction in the native bile.

significant data are included again for purposes of direct comparison.

The question arose as to whether urine alone was capable of converting mesobilirubinogen to stercobilinogen. Mesobilirubinogen was therefore added to 3 separate urobilin and urobilinogen-free samples, as shown in Table IV. After incubation for 3 days, the samples were acidified with sufficient HCl to make a concentration of approximately 5% and they were then extracted repeatedly with $CHCl_3$. The combined $CHCl_3$ was filtered and subjected to chromatographic analysis as described in Paper II. As noted in Table IV, urobilin IX,a was identified on the basis of optical inactivity and there was no evidence of stercobilin formation.

*Discussion.* The results of the present study based upon oral administration of mesobilirubinogen and upon *in vitro* incubation of mesobilirubinogen with feces, indicate that this substance is converted in part to stercobilin. A survey of the above data would indicate at first glance that the degree of conversion was not large. Other factors, however, must be taken into account. For one thing, meso-

TABLE IV.
Addition of Mesobilirubinogen to Normal Urine.

| | Amt urine in cc | Amt mesobilirubinogen in mg | Type of urobilin obtained |
|---|---|---|---|
| 1. | 100 | 64 | Urobilin IX,a* |
| 2. | 100 | 64 (+ ¼ g acholic feces) | ” IX,a* |
| 3. | 500 | 58 | ” IX,a* |

*Optically inactive in $CHCl_3$.

bilirubinogen is very easily oxidized so that a considerable portion of the added material in the above *in vitro* experiments may well have been changed before it could be converted to stercobilin. In the *in vivo* studies the factor of absorption cannot be assayed. In both instances considerable amounts of mesobilirubinogen appeared in the urine during the 24 hours after its administration. This was identified by the subsequent identification of urobilin IX,a from each. Another factor to be taken into account is the yield of stercobilin which one may expect especially when working with very small amounts of material. It will be noted that less than 1 mg of crystals were obtained in the control feces of Experiment 1, Table III, although 20 g of feces contained 33 mg of urobilinogen. Under the best of conditions and working with large amounts of feces, the yield of stercobilin is usually from 5-15 and rarely exceeds 20% of the initial urobilinogen. In this connection it is of interest that Fischer and Libowitzky[2] added 100 mg of mesobilirubinogen to a daily amount of feces and were able to recover but a very few crystals. This was not incubated and the possibility that some conversion to stercobilin had occurred was not mentioned. We have observed repeatedly that incubation of feces causes a sharp decrease in yield, so that the question arises in experiments of the above type as to whether much more stercobilinogen than was present at the time of extraction had been successively formed and oxidized during incubation. Most important, however, is the fact that significant increases of stercobilin were obtained, whose only source could have been the added mesobilirubinogen.

It is significant that in one of the present experiments (No. 3 in Table III), stercobilin formation was noted when mesobilirubinogen was incubated with urobilin-free bile and acholic feces. This is in contrast with the formation of d-urobilin in an entirely similar experiment (3c) in Paper III. Since this was carried out with bile and feces from different individuals, it can only be assumed that unknown and variable factors in bile or feces determine the direction of conversion.

The present results when correlated with those of the preceding papers indicate clearly that mesobilirubinogen is the primary chromogen or urobilinogen, and stercobilinogen and d-urobilinogen are to be regarded as secondary products, the former requiring both bile and fecal factors and the latter only a bile factor for its elaboration. This concept is perhaps best shown in the following diagram:

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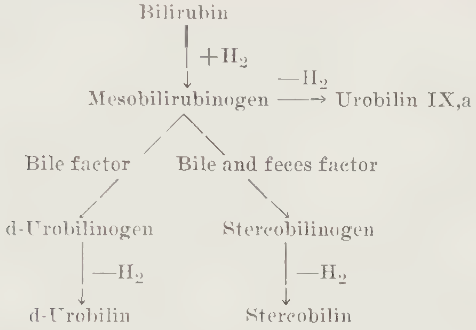
[2] Fischer, H., and Libowitzky, H., *Z. physiol. Chem.*, 1939, **258**, 255.



The primary reduction to mesobilirubinogen is probably effected by a number of bacterial reducing systems, while the secondary transitions to d-urobilinogen and stercobilinogen evidently depend on more complex factors, as indicated. The possibility cannot be excluded that bilirubin may be reduced directly to d-urobilinogen or stercobilinogen without mesobilirubinogen as an intermediate stage, also, that given the proper conditions, d-urobilinogen may be converted to stercobilinogen. Still another possibility is that stercobilin or d-urobilin may be formed directly from mesobilirubinogen without intermediary differing chromogens.

*Summary and Conclusions.* 1. The oral or intraduodenal administration of mesobilirubinogen resulted in an increased excretion of stercobilin in the feces. 2. The incubation of mesobilirubinogen with normal feces, or with acholic feces plus bile resulted in formation of stercobilin. Incubation with acholic feces alone resulted in no change; urobilin IX,a was subsequently isolated. 3. Mesobilirubinogen was not converted to stercobilin in the 3 urine samples studied. It is believed that mesobilirubinogen is the primary chromogen or urobilinogen formed by bacterial reduction of bilirubin. The further elaboration of d-urobilin requires bile, while that of stercobilin requires bile and feces.

## 13658

## Experimental Studies in Metaplastic Ossification.

SHELDON A. JACOBSON. (Introduced by Valy Menkin.)

*From the Laboratory of Pathology, Hospital for Joint Diseases, and the Richard Morton Koster Research Laboratory.*

Huggins[1-4] has discovered that transplanted epithelium of bladder, ureter, or kidney pelvis induces ossification of the connective tissue of muscle or muscle sheath in the dog. He further found that this epithelium possesses the same potency in *fascia lata,* subcutaneous tissue or synovial membrane, but not in kidney, liver or spleen. These experiments, successful in the dog, failed (except for one case) in the rabbit, despite the administration of viosterol and parathormone. Dragstedt and Kearns[5] and the author were able to reproduce his results. The former succeeded also in demonstrating ossification about a bit of fascia used to close a surgically produced defect in the bladder walls of dogs.

Sacerdotti and Frattin[6] found that if the vessels of one kidney of a cat, or one renal vein of a rabbit, were tied off, bone would be produced in the organ. It has been shown by Jaffe[7] and others that elementary phosphorus is a stimulus to osteosclerosis. Barth[8] reported ossification in the cat's omentum in response to the implantation of burned bone.

To study the mechanism involved more easily and on a larger scale, albino rats of both sexes and from 105 to 385 g in weight were selected. Under amytal anesthesia pieces measuring several mm in diameter were excised from the bladder and implanted into one or more pockets in the rectus muscle or elsewhere as mentioned. Variations of this and other procedures were employed as indicated in Table I. After a period of time varying from 3½ to 7 weeks, the animals were sacrificed, unless found dead. The experimental areas, when identifiable, were removed for microscopic section. Cysts resulted from the transplantations. They were lined by epithelium of the lower urinary type, varying markedly in thickness, and had many branching pockets. Besides the cysts, solid or almost solid clusters of epithelial cells often appeared. Granulation tissue surrounded the transplants, with lymphocytes and histiocytes in varying numbers. The occasional abscesses are not included in this report.

Three of the animals were found to be pregnant at autopsy. (None of these was among the 5 cases with positive results.)

The results are summarized in Table I. (The excess of number

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[1] Huggins, C. B., PROC. SOC. EXP. BIOL. AND MED., 1930, **27**, 349.

[2] Huggins, C. B., and Compere, E. L., PROC. SOC. EXP. BIOL. AND MED., 1930, **27**, 753.

[3] Huggins, C. B., PROC. SOC. EXP. BIOL. AND MED., 1930, **28**, 125.

[4] Huggins, C. B., *Arch. Surg.*, 1931, **22**.

[5] Dragstedt, C. A., and Kearns, J. E., Jr., *Am. J. Physiol.*, 1932, **100**, 262.

[6] Sacerdotti, C., and Frattin, G., *Gior. d. r. Accad. di Med. di Torino*, 1901, **7**, 825; *Arch. Path. Anat.*, 1902, **168**, 431.

[7] Jaffe, H. L., *Arch. Path.*, 1933, **16**, 63, 236.

[8] von Korff, K., *Birch. Arch.*, 1940, **276**, 111.

## TABLE I.

Metaplastic Ossification Experiments on Rats.

| Transplants (autoplastic unless otherwise stated) | Area of implantation | Supplementary treatment | No. animals | No. Exp. | Animals with cysts | No. instances of ossification |
|---|---|---|---|---|---|---|
| Bladder, segment | Rectus muscle | | 14 | 28 | 9 | 0 |
| ,, dome | ,, ,, | | 4 | 4 | 3 | 1 |
| ,, minced | ,, ,, | | 4 | 4 | 2 | 0 |
| ,, segment | Peritoneum | | 2 | 3 | 0 | 0 |
| ,, ,, | Spleen | | 2 | 2 | 0 | 0 |
| ,, ,, | Liver | | 1 | 1 | 0 | 0 |
| ,, ,, | Kidney | | 1 | 1 | 0 | 0 |
| ,, ,, | Rectus muscle | 1 cc $CaCl_2$ 1½% sub-cu. daily in graft area | 8 | 16 | 0 | 1 |
| ,, ,, | ,, ,, | Ca lactate ½% as drinking water | 2 | 4 | 1 | 0 |
| ,, ,, | ,, ,, | Viosterol 50 × normal conc. daily *per os* 0.1 cc | 9 | 18 | 7 | 1 |
| ,, ,, | ,, ,, | Viosterol 500 × normal conc. daily *per os* 0.1 cc | 2 | 4 | 0 | 0 |
| ,, ,, | ,, ,, | Elementary phosphorus 0.025 mg daily *per os* | 2 | 2 | 3 | 1 |
| ,, ,, | ,, ,, | Elementary phosphorus 0.05 mg daily *per os* | 1 | 2 | 0 | 0 |
| ,, ,, | ,, ,, | Radium emanation seed implanted in one kidney with variable degrees of destruction, elementary phosphorus 0.05 mg daily *per os*; kidney and transplant sites examined | 3 | 6 | 2 | 0 |
| Bladder and boiled rat bone | ,, ,, | | 2 | 4 | 1 | 0 |
| Bladder and boiled rat bone | ,, ,, | Viosterol 50 × normal conc. daily *per os* 0.1 cc | 2 | 4 | 1 | 0 |
| Bladder and boiled beef bone | ,, ,, | | 3 | 6 | 3 | 0 |
| Bladder and $AgNO_3$ crystal | ,, ,, | | 3 | 5 | 0 | 0 |
| Kidney segment | ,, ,, | | 1 | 1 | 0 | 0 |
| ,, cortex | ,, ,, | | 3 | 3 | 0 | 0 |
| ,, medulla | ,, ,, | | 3 | 3 | 1 | 0 |
| ,, pelvis | ,, ,, | | 7 | 13 | 3 | 0 |
| Ureter | ,, ,, | | 5 | 9 | 2 | 0 |
| Boiled rat femur | ,, ,, | | 2 | 4 | 0 | 0 |
| ,, beef bone | ,, ,, | | 3 | 6 | 1 | 0 |
| $AgNO_3$ crystal | ,, ,, | | 2 | 4 | 0 | 0 |
| Vessels of one kidney ligated | | | 4 | | | 1 |
| Dome of bladder removed and replaced by fascia | | | 5 | | | 0 |
| Dome of bladder removed, everted and replaced inside out | | | 1 | | | 0 |
| Dome of bladder removed, everted and replaced inside out | | Elementary phosphorus 0.025 mg daily *per os* | 1 | | | 0 |
| Totals | | | 102 | | | 5 |

of experiments over number of animals refers to multiple transplants.) Five experiments out of a total of 170 yielded evidence of metaplastic ossification. In each of these the bone was represented by a minute islet only.

Nine guinea pigs of both sexes and from 200 to 350 g in weight were next used for bladder transplants into the rectus muscle. At the end of a month, the animals were sacrificed. In other cases animals were initially sacrificed and their bladders cut up for homoplastic transplantation into 17 others. Takes were comparable in the two groups. Eighteen of the 26 cases manifested ossification at the transplant sites. Five were completely negative. In all 5, epithelium was absent. (It is, therefore, possible that the grafts did not take. This cannot be inferred, however, because bone was sometimes found when epithelium had disappeared.) The 3 remaining presented considerable areas of non-calcified ground substance.

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## 13659

## Action of Curare on the Brain of the Frog.

SERGEI FEITELBERG* AND ERNEST P. PICK.†

*From the Laboratories of the Mount Sinai Hospital, New York City.*

It is generally accepted that curare action is peripheral. An effect of curare on the brain has been occasionally assumed (Koelliker,[1] Steiner,[2] Meyer[3]), but never demonstrated conclusively.

We have attempted to present a definite proof of a central action of curare by the investigation of electroencephalograms on frogs.

*Procedure.* Frogs *(Rana esculenta)* were divided into 2 groups. One group was poisoned by curare (injection in the ventral lymph sac). Following this the electrical activity of the brain was investigated. The frogs of the second group were first pithed. After the electrical activity of the intact brain was determined, these frogs were poisoned by curare, and a second determination of the cerebral electrical activity was made. In a separate series of control experiments (12 frogs) it was ascertained that the electroencephalographic

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* Aided by a grant from the Dazian Foundation for Medical Research.

† Aided by a grant for apparatus by the Ella Sachs Plotz Foundation.

[1] Koelliker, A., *Virchow's Arch.*, 1856, **10**, 58.

[2] Steiner, J., *Das Amerikanische Pfeilgift Curare*, Leipzig, 1877, p. 36 f.

[3] Meyer, H. H., Gottlieb, R., and Pick, E. P., *Experimentelle Pharmakologie*, Vienna-Berlin, 1936, p. 36.

pattern of a pithed frog remains essentially unaltered for over 12 hours, if no drugs are administered (Fig. 1, first graph).

This method enabled us to trace the normal brain waves, and their alteration in the state of curare poisoning.‡

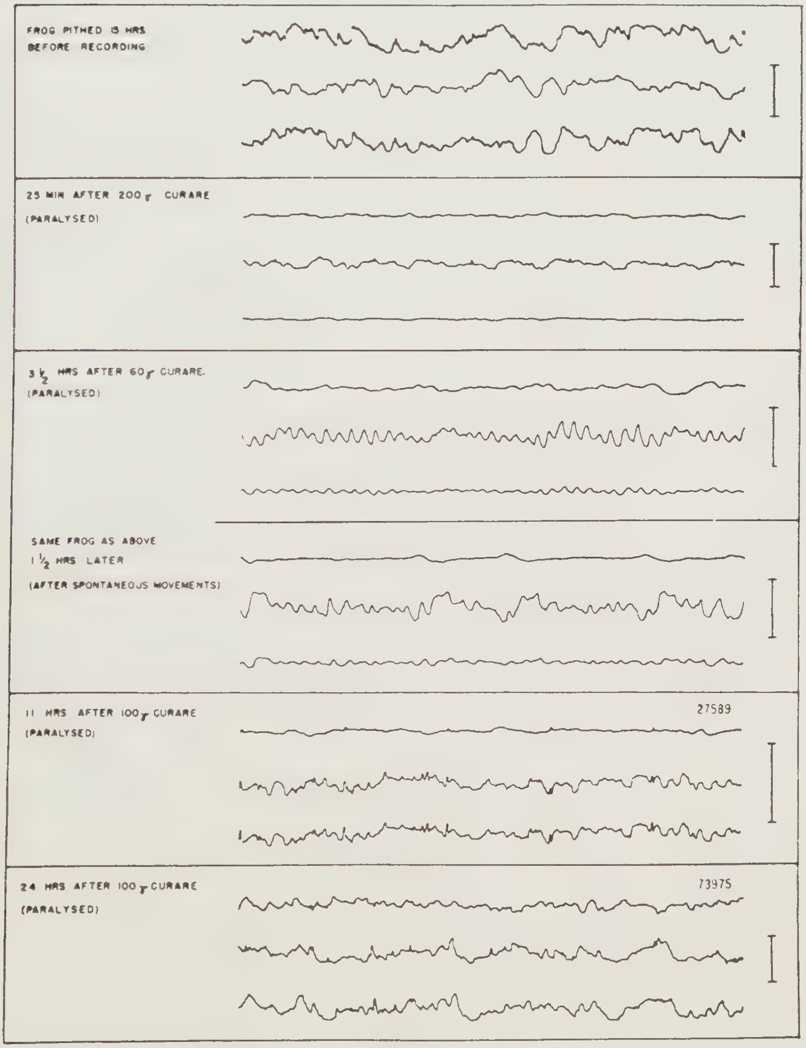

FIG. 1.

Electroencephalograms of frog. Length of each record—8 sec. Marks at right = 100 microvolt. All tracings from different frogs, except the third and fourth tracing.

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‡ The highly effective curare (80 γ produces total paralysis of a frog of average weight of 40 g in about 15 to 20 min) was kindly supplied by Dr. Claus Unna from Merck & Co., Rahway, N. J.

We used 3 Hoagland electrodes.[4] The localization of the 3 electrodes in the brain of each animal was uniform. The first lead was taken from the midpoint of the left hemisphere, the second was taken from the anterior portion of the right hemisphere, and the third lead was taken from the right optic lobe. The leads were paired in 3 channels, the first from the electrodes 1 and 2, the second from electrodes 2 and 3, and the third channel from electrodes 1 and 3. Bleeding was carefully avoided.

*Results.* Curare dosage which produces even temporary paralysis of the legs causes a gradual disappearance of the electrical brain potentials. Thirty-eight frogs have been examined, and an alteration of the electroencephalographic pattern was observed in each experiment. First the small fast waves (beta waves) disappear, leaving only the slower alpha waves. These very soon decrease both in frequency and amplitude. After some time (the interval depending on the dose) an electroencephalogram similar to that seen in deep narcosis results. The heart rate remains apparently undisturbed in this state of curare poisoning. This can be recognized by the electrocardiogram which is superimposed on the electroencephalogram. This curare narcosis may continue for 12 to 24 hours after injection of small amounts of curare, and then gradually changes into a state of complete recovery and reappearance of normal electrical potentials (Fig. 1). This may occur before or after reappearance of muscular motility (Fig. 1, compare the last 3 graphs).

*Conclusion.* Curare produces in frogs a brain narcosis simultaneous with a peripheral motor paralysis. The former may be recognized in the gradual alteration and final disappearance of electrical brain waves, and after a period of several hours and even days in a complete restoration of the normal electrical pattern. The peripheral and central actions are independent of each other.

We are indebted to Dr. Israel S. Wechsler for the use of the electroencephalographic apparatus.

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[4] Hoagland, H., *Science*, 1940, **92**, 537.

## 13660

# Heat Narcosis of Brain and of Striated Muscle of the Frog.

SERGEI FEITELBERG* AND ERNEST P. PICK.†

*From the Laboratories of the Mount Sinai Hospital, New York City.*

Claude Bernard observed that frogs that had been immersed in water of 37 to 38°C lost their reflexes and became completely paralyzed. After cooling, they regain their motility. Because of the reversible central paralysis in this experiment, Meyer[1] referred to it as "heat narcosis" and attributed it to changes in the lipoids, similar to those ascribed to other forms of narcosis. The widespread occurrence of lipoids in all cells of the body could, according to Meyer, satisfactorily account for paralysis of tissues other than those of the nervous system.

I. *The effect of heat on striated muscle.* The effects of heat have been demonstrated on the conduction system of the frog heart (Amsler and Pick,[2] Clark[3]), on the sciatic nerve of the frog (Bremer and Titeca[4]), and on the smooth muscle of the guinea pig uterus (Evans[5]). Because of the apparent generalized paralysis of frogs immersed in hot water, we have chosen to investigate skeletal muscle itself. The muscles of the left hind limb *(Rana esculenta)* were prepared for the experiment by section of the nerve trunks to this extremity as they left the vertebral canal. There was an interval of 3 weeks between the time of the nerve section and the experiment. After this period degeneration of the nerves is complete.

Such prepared frogs were immersed in water of 38 to 40°C for a period of 5 to 10 minutes, after which they were removed. This immersion produced heat paralysis. Electrodes were then inserted into the gastrocnemius muscle of the denervated extremity. A 60 cycle sinusoidal alternating current was used for stimulation. The magnitude of the threshold stimulus necessary to produce a twitch was determined every 2 to 3 minutes. It was found that the threshold

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* Aided by a grant from the Dazian Foundation for Medical Research.

† Aided by a grant for apparatus by the Ella Sachs Plotz Foundation.

[1] Meyer, H. H., "Waermenarkose," in *Experimentelle Pharmakologie,* by H. H. Meyer, R. Gottlieb, and E. P. Pick, 1936, Berlin and Vienna, 154.

[2] Amsler, C., and Pick, E. P., *Arch. f. Exp. Path. und Pharm.,* 1918, **84**, 234.

[3] Clark, A. J., *J. Physiol.,* 1920-1921, **54**, 275.

[4] Bremer, F., and Titeca, J., *Compt. rend, des séances de la Société de Biologie, Soc. Belge de Biol.,* 1933, **115**, 413, and 1934, **118**, 371.

[5] Evans, C. L., *J. Physiol.,* 1921, **54**, 139 (proceedings).

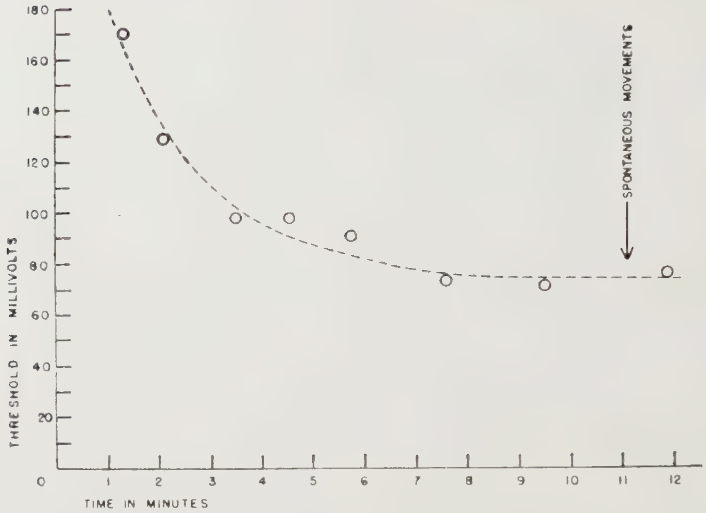

FIG. 1.

Threshold of the denervated gastrocnemius of the frog, after immersion for 10 min. in water of 39°C.

is highest immediately after removal of the frog from the warm bath, drops rapidly in the first few minutes and gradually returns to normal as the animal approaches room temperature. The normal threshold is usually reached before spontaneous muscular movements appear in the intact limbs. Fig. 1 illustrates this experiment.

II. *Effect of heat on the brain (heat narcosis).* Heretofore it has not been possible to demonstrate cerebral heat narcosis by direct qualitative and quantitative method. We have attempted to do this by means of electroencephalography.

Frogs were immersed in a water bath of 38 to 40°C for a period of 10 minutes. This produced complete paralysis. After removing the animals from the water bath three Hoagland electrodes were placed into the skull[6] and the electroencephalogram recorded continuously until the frogs regained spontaneous muscular activity (Fig. 2). At first, while the frog is completely paralyzed, the amplitudes of the electrical brain waves are very low, as in deep narcosis (1 min.). After a while short bursts of fast alpha waves (15 to 20 per second) appear (7 min.), and these bursts gradually become more prolonged and occur more often (10 min.) until they merge into a continuous pattern corresponding to the electroencephalogram of a normal frog (14 min). The frog recovers from his paralysis at this time. A similar series of events is observed following ether narcosis (Fig. 2).

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[6] Feitelberg, S., and Pick, E. P., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 654.

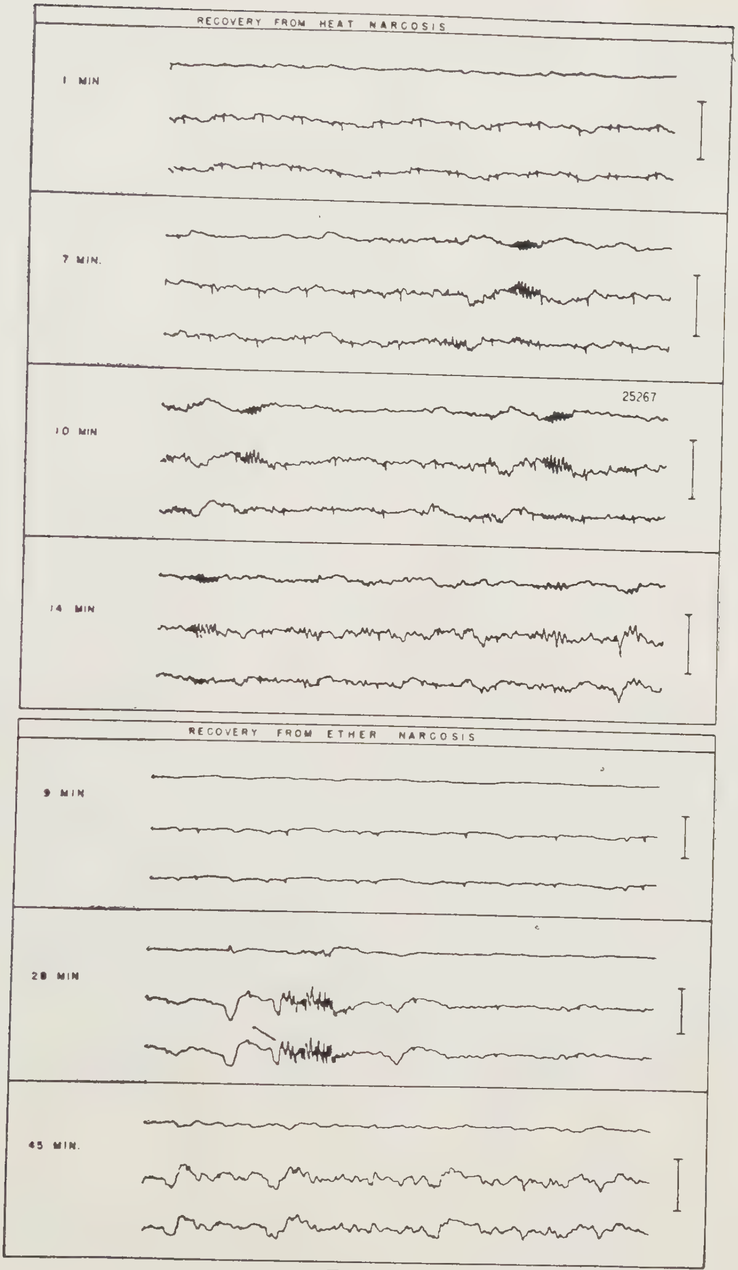

FIG. 2.

Electroencephalograms (frog) during recovery from heat and ether narcosis. Length of each record—8 sec. Marks at right = 100 microvolt. Notations at left indicate time after removal from water (40°C, immersion for 10 min.) and after removal of ether, respectively.

It appears that the respiratory center is the first to recover, since the alpha bursts described above coincide with the slight, but distinct respiratory movements which are the first manifestations of the return of muscular activity.

Heat narcosis as demonstrated by the electroencephalogram can also be produced by immersion in water of 33 to 35°C, although the frogs have to be immersed for a longer period of time (15 to 20 minutes) and the recovery is more rapid.

*Conclusion.* The effects of immersion of frogs in water of 38 to 40°C on the threshold of denervated skeletal muscle and on the electrical activity of the brain have been investigated. (1) The muscular threshold to electrical stimulation is increased, and returns to normal after 5 to 10 minutes. (2) The brain waves disappear almost completely. After several minutes fast alpha activity appears in single bursts, which occur more often. After 10 to 15 minutes the normal brain pattern returns. (3) The effects on the muscle and on the brain do not appear to be related with each other since the muscular threshold returns to normal before the appearance of a normal electroencephalogram.

The present experiments demonstrate for the first time that the brain is subject to a reversible heat paralysis as other organs.

## 13661 P

## Lobule-Alveolar Growth of Mammary Glands of Hypophysectomized Female Rats.

Ralph P. Reece and Samuel L. Leonard.

*From the Department of Dairy Husbandry, New Jersey Agricultural Experiment Station,* New Brunswick, N.J., and Department of Zoology, Cornell University, Ithaca, N.Y.*

In previous experiments we[1] have observed that the simultaneous administration of estrogen and growth hormone induced typical estrogen stimulation of the mammary glands of hypophysectomized male rats. In addition it has been shown that the injection of testosterone into sexually mature spayed rats stimulated growth of the lobule-alveolar system of the mammary glands[2] and furthermore in hypophysectomized male rats no growth occurred.[3] Consequently it was of interest to determine the effect of testosterone alone and in conjunction with the growth hormone on the mammary glands of spayed hypophysectomized rats.

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* Journal Series Paper of the New Jersey Agricultural Experiment Station, Rutgers University, Department of Dairy Husbandry.

[1] Reece, R. P., and Leonard, S. L., *Endocrinology*, 1941, **29**, 297.

[2] Reece, R. P., and Mixner, J. P., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 66.

Twenty sexually mature virgin rats were ovariectomized and hypophysectomized. Beginning on the day following operation 6 of the rats were injected subcutaneously daily for 15 days with 300 $\gamma$ of testosterone propionate[†] and the remaining 14 rats received subcutaneously 300 $\gamma$ of testosterone propionate plus 0.5 cc of growth hormone[‡] intraperitoneally daily for 15 days. The rats were sacrificed on the day after the last injection and the right abdominal mammary glands removed and studied as whole mounts. Completeness of hypophysectomy was checked by sectioning the *sella turcica*. The operation was complete in all animals included in this report.

The mammary glands of the testosterone-treated rats showed involutionary changes, although many lateral buds were still intact (Fig. 1). In marked contrast were the mammary glands of the rats receiving the combined treatment. The lobule-alveolar system had been developed, the development being especially extensive in the

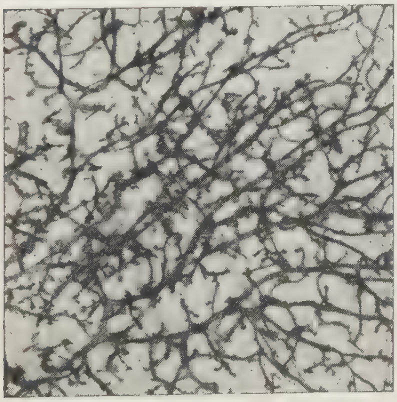

FIG. 1.

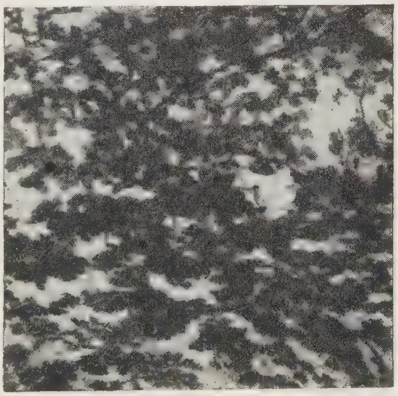

FIG. 2.

FIG. 1. Whole mount of a mammary gland of a spayed hypophysectomized rat injected with 300 $\gamma$ of testosterone propionate daily for 15 days.

FIG. 2. Whole mount of a mammary gland of a spayed hypophysectomized rat injected with 300 $\gamma$ of testosterone + 0.5 cc of growth hormone daily for 15 days.

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[3] Leonard, S. L., and Reece, R. P., *Endocrinology*, 1942, **30**, 32.

[†] The testosterone propionate (Perandren) was kindly supplied by Doctor R. MacBrayer of the Ciba Pharmaceutical Products, Inc., Summit, N.J.

[‡] The growth hormone (Squibb) was kindly supplied by Doctor J. S. De Frates of E. R. Squibb & Sons, New Brunswick, N.J.

proximal region of the gland (Fig. 2). The mammary glands of 12 out of 14 injected rats showed alveolar growth.

*Conclusions.* The injection of 300 γ of testosterone propionate daily for 15 days into spayed hypophysectomized rats failed to induce mammary growth. Lobule-alveolar growth of the mammary glands was induced in spayed hypophysectomized rats by the administration of 300 γ of testosterone propionate plus 0.5 cc of growth hormone daily for 15 days. Therefore some pituitary "mammogen" is essential for lobule-alveolar development of the rat mammary gland when stimulated by testosterone.

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13662

## Effect of Magnesium Deficiency on Dentin Apposition and Eruption in Incisor of Rat.

J. Gagnon, I. Schour and M. C. Patras.

*From the Department of Histology, College of Dentistry, University of Illinois, and the Department of Physiology, School of Medicine, Loyola University.*

Seventeen white rats (68-138 days of age) were fed the Kruse-Orent-McCollum diet,[1] which is deficient in magnesium (1.8 parts per million), for a period of 22-85 days. Eleven littermate controls were placed on the deficient diet to which magnesium was added in the form of $MgSO_47H_2O$. A group of 7 rats which were fed a modified form of this diet (the salt mixture was reduced one-half in amount) showed similar but less advanced effects.

*Radiographic Findings.* In the incisor the earliest change seen after 22-40 days on the deficient diet is a widening of the periodontal membrane, an indistinct outline of the *lamina dura* and a disturbed contour of the enamel surface. These changes become more marked with longer survival periods (50-76 days), when the pulp is shifted labially, with concomitant thinning of the dentin and folding of the basal portion. The pulp also shows radiopaque longitudinal streaks. The angle of the incisal bevel is increased from the normal of 45° to as high as 75°.

In the advanced stages the molars show a widened periodontal membrane and a rarefied appearance of the alveolar bone, so that the molar roots stand out clearly by contrast.

*Rate of Eruption.* In the deficient animals, the first 2 weeks show a

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[1] Kruse, H. D., Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, 1932, **96**, 519.

TABLE I.
Comparison of Mean Rates of Eruption During a Period of 12 Weeks of the Incisors of Normal Rats and Rats on Magnesium Deficiency.

| Group | No. of individuals | No. of measurements | Mean weekly eruption rate, mm | |
|---|---|---|---|---|
| | | | Upper Inc. | Lower Inc. |
| Normal control | 6 | 140 | 2.3 | 3.1 |
| Rats placed on magnesium deficiency | 7 | 140 | 0.9 | 1.3 |

decrease in eruption rate, after which a fairly constant low rate (about one-third the normal) is maintained (Table I).

Fig. 1-C contrasts the total increments of the eruption rates of control animal 18.26 and experimental animal 17.19, and shows the typical trend of the eruption rates.

*Rate of Formation.* Ground-section measurements of the rate of dentin apposition in the incisor by means of vital staining with alizarine Red S show that the rate of apposition is progressively decreased as the dietary deficiency is prolonged (Table II). The enamel-covered dentin is markedly retarded almost immediately, while the retarding effect is more gradual on the lingual dentin.

Apposition of dentin may cease for a time in any locality, and begin again later. This may be seen in sections where 2 alizarine lines join, indicating a lack of growth between succeeding injections (Fig. 1-A and D.2).

The difference in the dentin response is also seen in the total widths of the labial and lingual portions. These are approximately the same in the normal animal (Fig. 1-B and D.1). In the experimental animal the labial width is one-third or one-half that of the lingual width, and the pulpal outline, therefore, is characteristically distorted (Fig. 1-D.2). The response is in contrast to that found in

TABLE II.
Average Daily Rate of Apposition of Dentin of Incisors Based on Experiments with 10 Rats Placed on a Diet Deficient in Magnesium, and with 8 Controls.

| Age range in days | Experimental | | | Control | | |
|---|---|---|---|---|---|---|
| | No. of animals | Avg rate of apposition in $\mu$ | | No. of animals | Avg rate of apposition in $\mu$ | |
| | | Labial | Lingual | | Labial | Lingual |
| 45-60 | 6 | 8.2 | 14.3 | 2 | 19.2 | 18.3 |
| 60-75 | 10 | 6.0 | 11.4 | 3 | 16.2 | 17.8 |
| 75-90 | 7 | 3.7 | 8.7 | 5 | 15.7 | 16.7 |
| 90-105 | 4 | 2.6 | 4.5 | 6 | 15.7 | 14.2 |
| 105-120 | 2 | 2.7 | 4.2 | 5 | 16.3 | 14.3 |
| 120-140 | 1 | 1.2 | 1.0 | 3 | 16.3 | 13.4 |

Average age of animals at beginning of experiment was 47 days.

vitamin A deficiency where the enamel-covered dentin is accelerated and the cementum-covered dentin is decelerated in the rate of apposition[2] (Fig. 1-D.3). The difference between the labial and lingual portions in magnesium and in vitamin A-deficiency is sharply demarcated at the level of the cemento-enamel junction.

The rate of formation was also slowed in the dentin of the molars. In the alveolar bone, the rate of apposition was one-third to one-half the normal amount.

The food intake of the experimental animals is approximately the same in quantity as that of the control group and the animals gained in weight but at a slower rate. The striking retardation in rate of

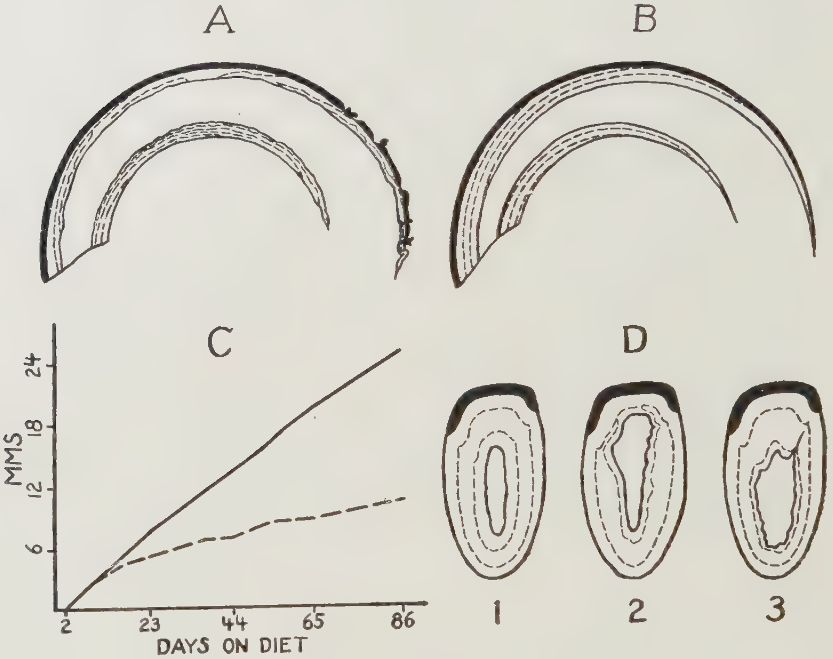

FIG. 1.

A. Diagrammatic representation of the irregular incremental pattern of the incisor of a rat placed on a diet deficient in magnesium and vitally stained with alizarine Red S. This figure was prepared by projecting and tracing the outline and alizarine lines (broken lines) of the incisor of rat 19.32.

B. Diagrammatic representation of the regular incremental pattern of control rat 18.26.

C. Graph showing total increments of eruption of upper incisors of control rat 18.26 (solid line) and magnesium-deficient rat 17.19 (broken line).

D. Diagrammatic representation of dentin growth in transverse section of the upper rat incisor as indicated by alizarine injections given 10 or 14 days apart in (1) normal; (2) magnesium deficiency; (3) vitamin A deficiency.

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[2] Schour, I., Smith, M. C., and Hoffman, M. M., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 447.

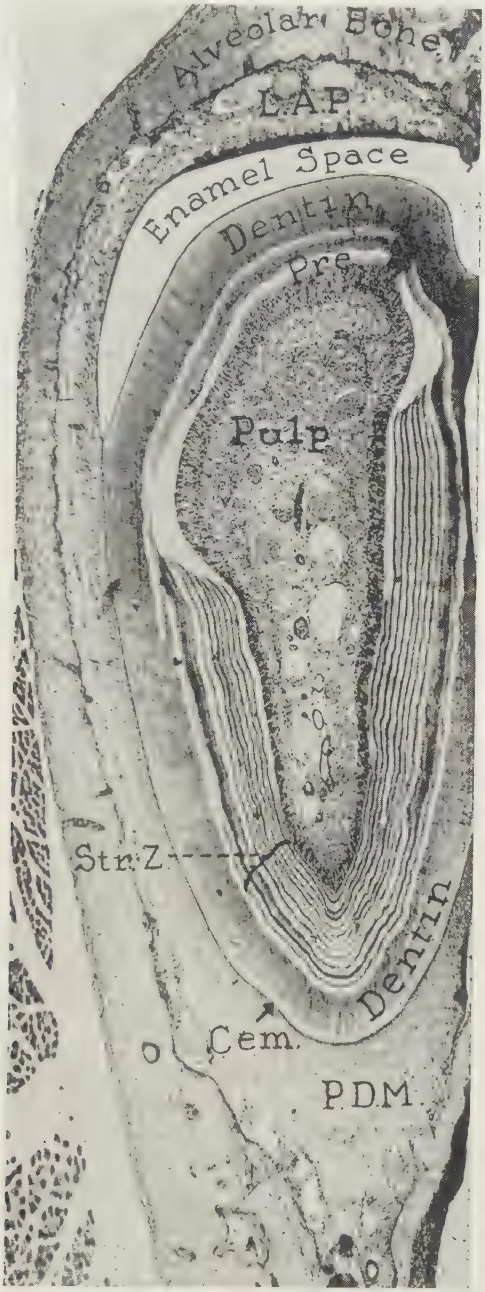

FIG. 2.

Photomicrograph of a transverse section of a lower incisor of a rat 109 days of age that was placed on a magnesium-deficient diet during the last 41 days of life. (×50.) Cem., cementum; L.A.P., labial alveolar periosteum; P.D.M., periodontal membrane; Pre., predentin; Str.Z., zone of prominent stratification.

dentin apposition and of tooth eruption is, therefore, not secondary to the relatively mild retardation in body weight. That the relationship between body weight and tooth development is not a primary one is also shown by the fact that normal animals subjected to insufficient food intake and retarded gain in body weight do not show significant effects upon tooth development.

*Calcification.* There is a characteristic disturbance of calcification rhythm in magnesium deficiency (Fig. 2). Irving[3] has reported this to be of a 2- or 3-day rhythm on the basis of measurements and comparison with the normal 16 $\mu$ daily rhythm. It appears that since apposition is slowed the 16 $\mu$ standard cannot be used. The difference between the calcification patterns of the labial and lingual dentin is probably linked with the difference in rate of apposition.

Enamel hypoplasia is not specific for Mg deficiency but is of a severe type (Becks and Furuta[4]).

*Summary and Conclusions.* In magnesium deficiency the rate of eruption of the incisor is reduced to approximately one-third the normal. The outstanding effect is on dentin apposition which is progressively decelerated, the enamel-covered dentin being affected more acutely than the cementum-covered portion. Temporary local cessations of growth may occur. The formation of alveolar bone is also decelerated to one-half or less of the normal rate and the periodontal membrane is abnormally wide. The dentin of the rat incisor acts not only as a kymograph of calcium metabolism (Fig. 2), but also as a growth kymograph (Fig. 1, D.2).

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### 13663

## Protyrosinase and Polar-Nonpolar Cations and Anions.*

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*From the Zoölogical Laboratory, State University of Iowa.*

The sodium alkyl sulfates, which activate protyrosinase,[1] yield polar-nonpolar anions, ($RSO_4^-$). Alkylamine hydrochlorides, however, give polar-nonpolar cations ($RNH_3.H^+$), and do not activate

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[3] Irving, J. T., *J. Physiol.*, 1940, **99**, 8.

[4] Becks, H., and Furuta, W. J., *J. A. D. A.*, 1941, **28**, 1083.

* Aided by a grant from the Rockefeller Foundation for research in cellular physiology.

[1] Allen, T. H., and Bodine, J. H., *Proc. Nat. Acad. Sc.*, 1941, **27**, 269.

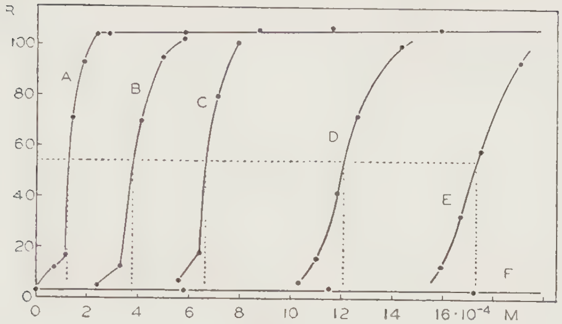

FIG. 1.

The effect at 24.9° of dodecylamine hydrochloride upon the activation of a constant amount of protyrosinase by sodium dodecyl sulfate. Ordinate: 1000 × the reciprocal of the time for the uptake of 100 μl of oxygen. Abscissa: molarity of sodium dodecyl sulfate for A, B, C, D, and E; molarity of dodecylamine HCl for F.

Curve A, sodium dodecyl sulfate.
B, " " " plus $0.96 \times 10^{-4}$ M dodecylamine HCl.
C, " " " " $1.91 \times 10^{-4}$ M " "
D, " " " " $3.82 \times 10^{-4}$ M " "
E, " " " " $5.73 \times 10^{-4}$ M " "
F, dodecylamine HCl.

protyrosinase.† This distinction may be of value in the search for the kind of chemical group or part of protyrosinase which is involved in its activation.

Three similar preparations of protyrosinase were extracted from grasshopper eggs *(Melanoplus differentialis)* by a method involving trituration, fractional precipitation, and dialysis.[1] Activities, measured in a Warburg apparatus, were compared by the reciprocal—multiplied by 1000—of the time in minutes for the first 100 μl of oxygen uptake with tyramine hydrochloride as substrate.

The ascending limb of curve A (Fig. 1) shows the activation of protyrosinase that occurs in the presence of sodium dodecyl sulfate. With dodecylamine hydrochloride (Curve F, Fig. 1) there seems to be no conversion of protyrosinase into tyrosinase. When solutions of alkyl sulfates and alkylamines are mixed, a precipitate forms. Thus, from activation experiments performed by adding a constant amount of protyrosinase to such mixtures (Curves B, C, D, E, Fig. 1), it was found at half activation that 1.00 mole of either decyl- or dodecylamine hydrochloride removes 0.36, 1.01, and 2.79 moles of sodium octyl, decyl, and dodecyl sulfates (Fig. 2). These values may well be a function of the dissociation constants of the compounds formed by cations and anions.

The effects of variation in the order of mixing of reagents is

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† The sodium alkyl sulfates were a gift from E. I. du Pont de Nemours; the alkylamine hydrochlorides were generously supplied by Dr. G. E. Boyd of the Department of Chemistry of the University of Chicago.

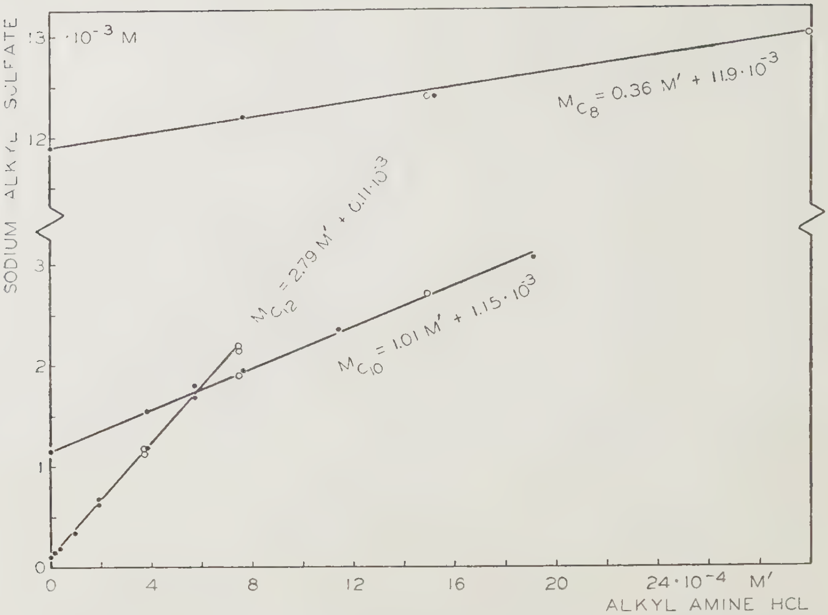

FIG. 2.

The effect of alkylamine hydrochlorides upon the activation of protyrosinase by sodium alkyl sulfates. Ordinate: molarity of alkyl sulfate needed for half activation. Abscissa: molarity of alkylamine hydrochloride mixed with alkyl sulfate at least 4 hours before adding protyrosinase. $M_{C_8} = 0.36M' + 11.9 \times 10^{-4}$ is the equation for the least squares line representing the effect of alkylamine hydrochlorides upon the activation of protyrosinase by sodium octyl sulfate. $M_{C_8}$, $M_{C_{10}}$, $M_{C_{12}}$ are the molarities of sodium octyl, decyl, and dodecyl sulfates. Open circles, decylamine HCl; closed circles, dodecylamine HCl. pH = 6.8, 0.033M phosphate buffer; temp. = 24.9° C. Reaction fluid volume = 3.0 ml.

graphically illustrated in Fig. 3. The upper curve (A) shows only a slight decrease in tyrosinase when excess dodecylamine is added *after* the mixing of protyrosinase and dodecyl sulfate. Curve B describes the effect, previously noted, of mixing dodecyl sulfate with dodecylamine and, *after* equilibration, adding the protyrosinase. Curve C refers to the results of mixing protyrosinase and dodecylamine and *then* adding the dodecyl sulfate. The difference between Curves A and B suggests that the activation of protyrosinase is irreversible, while the similarity in curves B and C would show that the dodecylamine cation not only fails to form a stable complex with protyrosinase but also reacts more readily with the alkyl sulfate anion than does protyrosinase.

In order to account for the drop of curve A (Fig. 3) a protyrosinase was heat-treated (5 min. at 70°).[2] The resulting tyrosinase, in the presence of increasing dodecylamine hydrochloride, showed a

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[2] Bodine, J. H., and Allen, T. H., *J. Cell. and Comp. Physiol.*, 1938, **12**, 71.

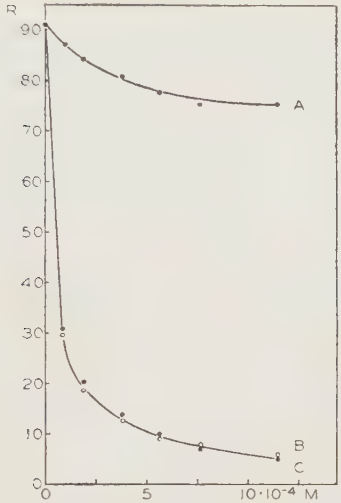

FIG. 3.

Variation in the order of mixing 1.59 × $10^{-4}$M sodium dodecyl sulfate, various amounts of dodecylamine HCl, and a constant amount of protyrosinase. Ordinate: specific activity (see Fig. 1). Abscissa: molarity of dodecylamine HCl. For description of Curves A, B, C see text.

similar drop in activity. Since this inhibition is independent of the method of activation, it seems that only *free* alkylamine cations are poisons for this tyrosinase. Yet, the alkylamine did not form a stable complex with protyrosinase (Curve C. Fig. 3). Apparently the alkylamine cation will react with tyrosinase but not with protyrosinase. If an ammonio-copper complex were formed between the copper[3] of this tyrosinase and the polar group of the alkylamine, it seems of interest to note that such a complex is apparently not formed with the copper[4] of protyrosinase.

It is evident that the polar groups of alkyl sulfates and alkylamines will combine with each other (Fig. 2). This fact in itself offers a suggestion as to the locus of activation. Perhaps an alkyl sulfate ion reacts with the amino group of a diamino acid belonging to a protyrosinase molecule. Since the intercept constants (Fig. 2) decrease with increasing size of the alkyl group thus introduced, it seems necessary to conclude that the efficiency[1] of activation is markedly increased by the addition of carbon atoms to the nonpolar group.

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[3] Bodine, J. H., and Allen, T. H., *J. Cell. and Comp. Physiol.*, 1941, **18**, 151.

[4] Allen, T. H., and Bodine, J. H., *Science*, 1941, **94**, 443.

### 13664

## Effect of Anoxemia on Myocardium of Isolated Heart of the Dog.

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*From the Departments of Biological Chemistry and Pharmacology, Harvard Medical School, Boston.*

Although the ultimate effects of anoxemia on tissue are clearly disorganization and death, the steps by which this final disorientation is produced have not been established. The heart-lung preparation seemed to be well suited to the study of the effects of anoxemia on the electrolytes of the heart. It has previously been shown that the production of temporary ischemia in localized portions of the heart of an intact animal results in a subsequent increase in the extracellular ions, sodium and chloride, and in water in the affected regions.[1] To test the effects of anoxemia rather than of ischemia, it would be desirable to reduce the oxygen supply to the heart without interfering with the circulation of blood. Although this could be accomplished with the intact animal, serious complications might result from the effects of anoxemia on the rest of the body. The use of the heart-lung preparation avoids these difficulties.

Observations were made on the electrolytes of the myocardium of the dog taken from heart-lung preparations in (a) 2 control experiments lasting about 1 hour, (b) 2 experiments in which the oxygen supplied to the lung of the heart-lung preparation was partially or completely replaced by nitrogen, and (c) 2 experiments in which the myocardium was poisoned, in one instance with sodium amytal and in the other with potassium.

The original electrolyte data have been used to calculate the relative amounts of the extra- and intracellular portions of the myocardium, and the concentrations of water, potassium, magnesium, and bicarbonate, as well as the pH in the cardiac fibers themselves. This type of histochemical calculation has become a well established procedure.

The electrolyte changes observed following an extended period of partial anoxemia were comparable to those produced following temporary ischemia in the intact animal. There is no evidence of significant change in the electrolytes of the fibers themselves.

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[1] Hastings, A. B., Blumgart, H. L., Lowry, O. H., and Gilligan, D. R., *Trans. Assn. Am. Physicians*, 1939, **54**, 237.

*Experimental.* The heart-lung preparation was made according to Patterson and Starling's technic[2] with certain modifications which have been previously described.[3] Chloralose, 0.09 g per kg body weight, was used as anesthetic for the operation. Defibrinated blood served as perfusion fluid.

*Gas Mixture.* The gas supplied to the lungs of the preparations consisted of (a) 5% $CO_2$/95% air in one control experiment, (b) air alone in the second control experiment and in the experiments with amytal and potassium poisoning, (c) 5% $CO_2$/95% $N_2$ in the first anoxemia experiment, and (d) 2% $O_2$/98% $N_2$ in the second.

At the end of each experiment, a sample of blood was centrifuged under paraffin oil and the serum saved for the determination of pH, chloride, sodium, total $CO_2$, and water.

Separate samples of myocardium were taken rapidly from the right and left ventricles at the termination of the experiment, while the heart was still beating. The tissue was analyzed for blood, fat, total $CO_2$, chloride, sodium, potassium, calcium, and magnesium. The determinations of blood, fat, chloride, sodium, and potassium were performed essentially as previously described.[4] The tissue $CO_2$ was measured by the method of Danielson.[5] The calcium was determined acidimetrically after precipitation as the oxalate and conversion to the carbonate. Magnesium was determined colorimetrically from the phosphate in the magnesium ammonium phosphate precipitate.

The procedures used for the calculation of data of histochemical interest have been described previously.[4]

*Results.* In Table I are shown the electrolyte and water concentrations observed in the myocardium in 5 experiments. All of the results are reported on a blood-free, fat-free basis. For comparison, average values obtained from the myocardium of 14 adult dogs are included in the table. These data have been reported previously.[1] The average values for bicarbonate and pH are from unpublished data of Dr. W. M. Wallace on the hearts of 3 young dogs.

*Control Experiments.* In the tissue obtained from the control experiments, the electrolyte pattern probably falls within the normal range. However, the sodium, chloride, and water concentrations approach the upper limit of normal, indicating an amount of extra-

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[2] Patterson, S. W., and Starling, E. H., *J. Physiol.*, 1913, **48**, 357.

[3] Krayer, O., *Arch. f. exp. Path. u. Pharmakol.*, 1931, **162**, 1.

[4] Lowry, O. H., and Hastings, A. B., *J. Biol. Chem.*, 1942, **143**, 351.

[5] Danielson, I. S., and Hastings, A. B., *J. Biol. Chem.*, 1939, **129**, 547.

TABLE I.

$E_{Cl}$, $E_{Na}$, and observed data (except $pCO_2$), reported per kg of blood-free, fat-free tissue, $(H_2O)_c$ reported per kg of fibers, and $[K]_c$, $[Mg]_c$ and $[HCO_3]_c$ reported per kg of fiber $H_2O$.

| | | Observed data | | | | | | | | | Derived data | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Experiment | | $H_2O$ g | Cl mE | $HCO_3^-$ mE | Na mE | K mE | Ca mE | Mg mE | Total base mE | $pCO_2$ mm | $E_{Cl}$ g | $E_{Na}$ g | $(H_2O)_c$ g | $[K]_c$ | $[Mg]_c$ | $[HCO_3^-]_c$ | $pH_c$ |
| Control | Serum | 924.2 | 115.2 | 12.33 | 145.2 | | | | | 41.3 | | | | | | 13.4* | 7.10† |
| 5% $CO_2$/ | L.V. | 795.6 | 30.8 | | 38.2 | 92.8 | 1.62 | 22.4 | 155 | (41.3) | 265 | 266 | 753 | 166 | 40 | | |
| 95% air | R.V. | 797.5 | 32.5 | 10.54 | 42.3 | 95.3 | | | | (41.3) | 284 | 298 | 755 | 176 | | 13.2 | 7.14 |
| Control | Serum | 924.2 | 121.8 | 8.45 | 146.5 | | | | | 12.2 | | | | | | 9.1* | 7.47† |
| air | L.V. | 786.1 | 30.2 | 10.07 | 41.4 | 95.8 | 1.47 | 20.5 | 159 | (12.2) | 247 | 285 | 741 | 174 | 37 | 14.5 | 7.71 |
| | R.V. | 793.5 | 32.6 | 8.98 | 39.8 | 92.0 | | | | (12.2) | 270 | 278 | 754 | 166 | | 12.2 | 7.64 |
| 5% $CO_2$/ | Serum | 917.3 | 116.3 | 14.28 | 148.3 | | | | | 38.5 | | | | | | 15.5* | 7.20† |
| 95% $N_2$ | L.V. | 800.0 | 30.0 | 9.66 | 38.8 | 83.4 | 1.62 | 21.3 | 145 | (38.5) | 255 | 262 | 761 | 146 | 39 | 10.5 | 7.10 |
| | R.V. | 795.5 | 31.8 | 11.27 | 40.0 | 89.0 | | | | (38.5) | 273 | 274 | 757 | 156 | | 13.4 | 7.17 |
| 2% $O_2$/ | Serum | 914.9 | 115.6 | 6.68 | 145.7 | | | | | 9.3 | | | | | | 7.3* | 7.49† |
| 98% $N_2$ | L.V. | 802.9 | 36.6 | 6.44 | 45.6 | 83.6 | 1.82 | 21.3 | 152 | (9.3) | 309 | 312 | 752 | 159 | 40 | 8.3 | 7.58 |
| | R.V. | 801.1 | 37.7 | 6.86 | 43.8 | 85.7 | | | | (9.3) | 322 | 304 | 754 | 164 | | 8.2 | 7.58 |
| Potassium | Serum | 915.6 | 114.7 | 5.78 | 141.4 | | | | | 8.7 | | | | | | 6.3* | 7.46† |
| poisoning | L.V. | 780.0 | 29.6 | 7.36 | 28.3 | 100.2 | 1.41 | 23.4 | 153 | (8.7) | 254 | 203 | 743 | 171 | 40 | 11.4 | 7.75 |
| air | R.V. | 779.5 | 29.7 | 7.53 | 30.7 | 101.0 | | | | (8.7) | 259 | 223 | 744 | 175 | | 10.9 | 7.73 |
| Normal dog | L.V. | 785.9 | 25.4 | 13.3§ | 31.1 | 89.0 | | | | | 220 | 223 | 750 | 152 | | 13.5§ | 7.09§ |
| | σ | 6.3 | 3.0 | | 4.3 | 2.4 | | | | | 17 | 19 | 7 | 4 | | | |
| myocardium‡ | R.V. | 789.1 | 26.6 | | 32.8 | 94.8 | | | | | 232 | 242 | 754 | 165 | | | |
| | σ | 7.5 | 2.5 | | 4.1 | 6.0 | | | | | 18 | 26 | 8 | 10 | | | |

*Concentration per kg serum $H_2O$.

†pH of serum.

‡Data previously published.[1]

§Unpublished data of Dr. W. M. Wallace on 3 young dog hearts.

cellular tissue, $E_{Cl}$ or $E_{Na}$, a little above the average for the heart of the intact dogs. The total base concentrations, estimated as the sum of the individual cations, averaged 157 mE per kg of tissue, or 208 mE per kg of fiber water.

The bicarbonate concentrations in the tissue as a whole, and in the fibers themselves, differed but little in the 2 control experiments in spite of a marked difference in the $CO_2$ tensions of the respective sera.

*Anoxemia.* Complete replacement of oxygen by nitrogen in the gas supplied to the heart-lung preparation resulted in failure of the heart within 15 minutes. Actually, complete anoxemia probably obtained for less than 10 minutes since some time was required for the lungs to blow off all of the oxygen in the blood of the preparation. This heart showed little change except for a moderate decrease in potassium in the left ventricle (Table I). In the belief that this heart may have stopped too promptly to allow electrolyte rearrangements to take place, another preparation was given a gas mixture consisting of 2% $O_2$/98% $N_2$ which permitted the heart to beat for an hour, at which time fibrillation occurred. This heart showed distinct increases in water, sodium, and chloride, and a small decrease in potassium. Calcium and magnesium were not significantly altered. From a histochemical interpretation of these data, there appears to have been an increase in the extracellular portion of the myocardium amounting to about 20% when compared to the controls. The concentrations *in the fibers* of potassium, magnesium, and water, $(H_2O)_c$, do not appear to be significantly altered by this sublethal degree of anoxemia. Similarly, the bicarbonate concentrations in the fibers, and the fiber pH are comparable in the control and hypooxemia experiment when due allowance is made for differences in the composition of the respective sera. Apparently as long as the heart is able to continue beating, the degree of anoxemia has not been sufficiently severe or prolonged to destroy the original electrolyte configuration in the fibers.

*Effects of Poisoning.* The injection of 20 cc of isotonic KCl during the course of an hour into a heart-lung preparation brought the serum level of potassium to 9.5 mE per liter, and nearly stopped the heart. In the myocardium, an almost normal electrolyte configuration was observed (Table I). Sodium appeared to be somewhat decreased in comparison with the chloride, and a somewhat increased potassium concentration was found. The possibility exists that the small amount of intracellular sodium originally present had been replaced by potassium, although the concentration of potassium within the fibers did not appear to be greatly increased.

The administration of 160 mg of sodium amytal in divided doses during a period of an hour to another preparation nearly stopped the heart, and produced slight changes in the electrolyte concentrations in the heart that were very similar to those produced by the potassium.

It would appear, therefore, that with decreasing amounts of oxygen, or with increasing amounts of potassium or amytal, the general impermeability of the cardiac fibers to electrolytes is maintained at least as long as the heart continues to beat.

*Summary.* 1. The myocardium from a heart-lung preparation respiring air or a mixture of 5% $CO_2$ and air presents a nearly normal electrolyte pattern. 2. Heart failure from oxygen deficiency in such a preparation results in an increase in sodium, chloride, and water, and a decrease in potassium of the myocardium. 3. These changes are interpreted as indicating an extracellular edema without significant change in the fibers themselves. 4. Sufficient potassium or amytal to produce incipient heart failure produced only small changes in the cardiac electrolytes.

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## 13665

## Urinary Gonadotrophins in Normal Men.

HERBERT M. EVANS AND AUBREY GORBMAN.

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There is a dearth of information as to the gonadotrophins found in the urine of normal men. This is to some extent due to the difficulty of preparing non-toxic concentrates, for high doses are necessary to reveal the low titer of gonadotrophins characteristic of normal male urine. Recently investigators employing alcohol, tannic acid, or tungstic acid as precipitation agents, appear to be agreed that the level of excretion of gonadotrophins in normal male urine is approximately 5 to 25 mouse-uterus units[1] per liter.

Witschi[2] has pointed out that the ratio of follicle stimulating to luteinizing or interstitial cell stimulating activities in any given gonadotrophin may be characteristic of that gonadotrophin. Fraenkel-Conrat, Li, Simpson and Evans,[3] using hypophysectomized rats have determined such ratios for various gonadotrophins having different hypophyseal and body-fluid sources, including one preparation from normal male urine. These workers found in an alcoholic precipitate approximately equal amounts of FSH and urinary ICSH. Leathem and Levin[4] in a similar assay, found that their tannic acid precipitate produced only follicular stimulation over a wide range of dosages, yet luteinization in the highest dosages.

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[1] Levin, L., and Tyndale, H. H., PROC. SOC. EXP. BIOL. AND MED., 1935, **34**, 516.

[2] Witschi, E., *Endocrinol.*, 1940, **27**, 437.

The present study was undertaken to provide an accurate determination of the FSH :ICSH ratio in the urine of normal men, since the published data are in apparent disagreement. In addition the effort was made to estimate quantitatively the amount of FSH and ICSH in a unit volume of urine, since such data do not exist.

*Procedure.* Pooled samples of the urine of college men were precipitated in lots of 20 to 30 liters. All extracts were assayed for quantity of hormone in 25-day-old female rats, or in 21-23-day-old female Swiss white mice. The endpoint in rats was the production of large follicles in the ovary and/or estrous uteri. The 100% increase in uterine weight was considered the endpoint in mice. The extracts were assayed, in addition, in hypophysectomized female rats, 26 to 28 days old at operation, first injected 6 to 8 days after operation. In these animals the urine dosage levels at which interstitial tissue repair (M.E.D. for ICSH), follicle stimulation (M.E.D. for FSH) or luteinization occurred were determined by histological studies.

All dosages were administered subcutaneously on 4 successive days. Autopsy was performed on the fifth day. In working with urines containing only a few units of hormone per liter it is essential that a dependable procedure giving complete hormonal recovery be properly employed. We have used a modified alcoholic precipitation technic, which in our hands has proven more satisfactory than tannic acid or tungstic acid precipitation.

After adjusting the pH of the urine to 4.0 to 5.0, the precipitation was made by addition of 4 volumes of 95% ethyl alcohol. The precipitate was allowed to settle out in the cold room at 4°C. The clear supernatant fluid was siphoned off and the precipitate quantitatively transferred to centrifuge cups. After washing twice with 95% alcohol, and once with ether, the precipitate was dried. The dry powder was placed in a celloidin bag (Visking casing) and the traces of powder remaining in the centrifuge cups were washed into the bag. After dialysis for 3 to 4 days at 4°C the supernatant in the bag was separated from any sediment by centrifugation. The supernatant was then frozen and desiccated under high vacuum while still in the frozen state. The powder so obtained was then weighed and aliquot parts were used for the assays. Levin[5] has pointed out that previous workers who have used the alcohol method tried to extract the hormone from the copious alcohol precipitate with small volumes of water. Since the water rapidly becomes saturated with urea and salts, this extraction is probably not complete. Solution of the hormone within the dialysis bag overcomes this objection. Secondly, drying of the extract while it is in the frozen state eliminates the loss of hormone which Levin indicates may occur during drying at room temperature.

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[3] Fraenkel-Conrat, H., Li, C. H., Simpson, M. E., and Evans, H. M., *Endocrinol.*, 1940, **27**, 793.

[4] Leathem, J. H., and Levin, L., *Anat. Rec.*, 1941, **79**, suppl., 42.

*Results.* Quantitative. In 10 samples of pooled normal male urines the titer of hormone ranged from 1.25 to 4.5 rat units of gonadotropic activity per liter, or 5.0 to 20.0 mouse-uterus units (Table I). These values agree well with those obtained by Saethre,[6] Katzman,[7] and Levin.[5] In general, where both rat and mouse assays were made for the same urine, one rat unit was equal to 4 to 6 mouse units. This 5-fold greater sensitivity of the mouse over the rat has been found to hold in assays of hypophyseal gonadotrophin, menopause urinary gonadotrophin, or other gonadotrophins relatively rich in the follicle stimulating factor. In assays of the urinary interstitial cell stimulating factor (pregnancy, chorioepithelioma) the rat has been found from 2 to 5 times as sensitive as the mouse.

*Results.* Qualitative. In all instances in which it was possible to determine the minimum dosages for follicle stimulation (FSH) and interstitial cell repair (ICSH) in the hypophysectomized rat, it was found that these effects appeared at approximately equal dosage levels. Although ICSH activity at no time appeared at dosages lower than those required for follicle stimulation, it was never necessary to give more than 2 to 3 times the M.E.D. for FSH to produce repair of interstitial tissue. At high dosages luteinization of follicles and formation of corpora lutea were seen. In this respect our results are in accord with those of Leathem and Levin[4] who, however, do not mention the interstitial cell repair which must have occurred at lower dosage levels.

*Discussion.* Leathem and Levin[4] state that as far as effects on

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[5] Levin, L., *J. Biol. Chem.*, 1940, **133**, suppl., lx.

[6] Saethre, H., *Klin. Wchnschr.*, 1935, **14**, 376.

[7] Katzman, P. A., *Endocrinol.*, 1937, **21**, 89.

TABLE I.
Summaries of Gonadotropic Assays of Concentrates from Normal Male Urine.

| Pooled urine preparation Sample No. | Method of precipit. | MED in rats equiv. cc urine | MED in mice equiv. cc urine | MED for FSH in hyp. rat,* cc urine | MED for ICSH in hyp. rat,* cc urine | Ratio ICSH:FSH (approx.) |
|---|---|---|---|---|---|---|
| WN 9 | alcohol | 500 | 80 | 460 | 690 | 1:1.4 |
| WN 28 | ” | 400 | 80 | 1330 | — | |
| WN 29 | ” | 250 | 50 | 400 | 500 | 1:1.2 |
| WN 30 | .8 sat. $(NH_4)_2SO_4$ | 500 | 120 | | | |
| WN 32 | alcohol | 570 | 140 | | | |
| WN 33 | tungstic acid | 800 | 160 | 1000 | — | |
| WN 34 | tannic acid | — | 200 | 2450 | — | |
| WN 35 | alcohol | 225 | — | 350 | 930 | 1:2.7 |
| WN 50 | ” | 400 | 80 | — | | |

*The MED for FSH is the minimal total dose which causes resumption of follicular development in rats hypophysectomized at 26-28 days of age, and first injected 6 to 8 days after operation. Injections are made subcutaneously for 4 days, once each day, and autopsy is performed on the fifth day. The MED for ICSH in similarly treated rats is that total dose which will cause repair of the ovarian interstitial tissue.

the ovaries of hypophysectomized rats are concerned, the urinary gonadotrophins of normal men resemble those found in normal women at or after the menopause. In the 3 samples of normal male urine, for which complete analyses are reported here, the ratio of urinary ICSH to FSH was 1:1.2, 1:1.4, and 1:2.7. It appears therefore, that it may not be strictly valid, at least when speaking of alcohol concentrates, to liken the normal male gonadotrophin to menopause gonadotrophin, where this ratio is 1:5 or 1:6.[3] The data of Fraenkel-Conrat, *et al.*, who reported the assay of one alcoholic precipitate of male urine in hypophysectomized rats and found a 1:1 ratio agree fairly closely with 2 of the samples reported here.

The slight difference in the 2 types of results described by Leathem and Levin on one hand and Fraenkel-Conrat, *et al.*, and this paper on the other hand, may be due to the difference in concentration technics. It is possible, since the difference may be referable to a loss of ICSH in the former case or a loss of FSH in the latter case, that one of the technics allows a selective loss of one of the hormones. That this is probably not true of the alcohol concentrates reported here is indicated by the normal quantitative titer obtained by assaying the same concentrates in normal rats or mice. Leathem and Levin report only the approximate ratio of FSH and LH in their tannic acid preparations but give no indication of the potency of these preparations expressed in terms of equivalent volumes of urine.

*Summary.* A modified alcoholic precipitation method is described for the preparation of non-toxic gonadotrophic concentrates from normal male urine.

Assayed in normal rats, such concentrates of pooled samples of urine of healthy college men give a quantitative titer of 1.0 to 4.5 rat units per liter. In normal mice titers of 6.0 to 20.0 mouse-uterine units per liter were obtained.

Assayed in hypophysectomized rats it is found that follicle stimulation and interstitial cell repair are usually produced at the same dosage level, although in some instances follicle stimulation may be produced at a dosage level as low as one-third that necessary for interstitial cell repair.

### 13666

## Qualitative Differences between Positive Streptococcal Antifibrinolysin Tests.*

PAUL L. BOISVERT. (Introduced by Grover F. Powers.)

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Positive streptococcal antifibrinolysin tests[1, 2, 3] are generally regarded as indicative of the presence of a specific "antibody." There are instances, however, when this seems unlikely.[4] Milstone[5] has recently demonstrated that the plasma-clots of rabbits are resistant to lysis by human strains of hemolytic streptococci because they lack a substance—"lytic factor"—which is present in normal human serum. The addition of "lytic factor" to rabbit plasma results in rapid liquefaction of the clot by fibrinolysin. This observation was of considerable interest to us because of its possible clinical application since it might explain some of the apparently non-specific positive antifibrinolysin tests (resistant clots) which we have observed in infants and children.[4]

Newborn blood frequently gives a positive test in spite of the

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* Aided by a grant from the Fluid Research Fund of the Yale University School of Medicine.

[1] Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

[2] Tillett, W. S., Edwards, L. B., and Garner, R. L., *J. Clin. Invest.*, 1934, **13**, 47.

[3] Tillett, W. S., *J. Clin. Invest.*, 1935, **14**, 276.

[4] Boisvert, P. L., *J. Clin. Invest.*, 1940, **19**, 65.

[5] Milstone, H., *J. Immunol.*, 1941, **42**, 109.

fact that there is no clinical or bacteriological evidence of a recent hemolytic streptococcal infection in the mother and her test is negative.[6,4]

As a starting point for the present study the blood from newborn infants was chosen first for comparison with the blood of children with positive tests following a hemolytic streptococcal infection. It seemed likely that newborns would generally have resistant plasma-clots for only one of two reasons—either because of absence of a substance or because of the presence of antifibrinolysin. In sick children both of these factors might be involved.

This study represents a comparison of the blood of 10 normal newborns with that of 10 children who were convalescing from scarlet fever and other hemolytic streptococcal infections. All had maximally positive streptococcal antifibrinolysin tests.

*Methods.* The routine antifibrinolysin test was performed as originally described by Tillett and Garner. 0.2 cc of human plasma was diluted with 0.8 cc of normal salt solution. To this was added 0.5 cc of an 18-hour broth culture of hemolytic streptococcus (known to dissolve normal plasma-clots in 15 minutes). Lastly, 0.25 cc of calcium chloride was added. The contents of the tube were thoroughly mixed and incubated in a waterbath at 37.5°C. A solid clot formed in about 10 minutes. The interval between the time of clotting and that of complete liquefaction determined the reading of the results of the test: 4+, no lysis of the plasma-clot in 24 hours; 3+, complete lysis in 8-24 hours; 2+, complete lysis in 3-8 hours; 1+, complete lysis in 1-3 hours; —, complete lysis in less than 1 hour.

In this study the test was varied to include serum, and the amount of saline was reduced accordingly so that the total volume of constituents remained the same. Serum was added just prior to the addition of calcium chloride since earlier addition of serum resulted in premature clotting. Normal serum was added in 0.8 cc amounts, and serum from newborns and from patients with a hemolytic streptococcal infection in 0.2 cc quantities.

TABLE I.

Blood of Newborns Lacks a Property Present in Normal Blood.

Newborn Plasma (by Routine Test) = Resistant Clot
'' '' + Normal Serum = Susceptible ''
'' Serum + '' Plasma = '' ''

Note: Resistant clot denotes no lysis of clot in 24 hours. Susceptible clot denotes complete lysis within one hour.

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[6] Lippard, V. W., and Wheeler, G. W., *Am. J. Dis. Child.*, 1936, **52**, 61.

TABLE II.
Blood of Streptococcal Convalescents Possesses a Property Absent from Normal Blood.

Streptococcal Convalescent Plasma (by Routine Test) = Resistant Clot
” ” ” + Normal Serum = ” ”
” ” Serum + ” Plasma = ” ”

Note: Resistant clot denotes no lysis of clot in 24 hours. Susceptible clot denotes complete lysis within 1 hour.

The results which are summarized in Tables I and II indicate that the resistant clots of newborns depend on a different mechanism than those observed in patients recovering from a hemolytic streptococcal infection. In newborns resistance is apparently due to the *absence* of a substance since their clots are rapidly dissolved when normal serum has been added, and resistance cannot be passively transferred to a normal plasma by the addition of newborn serum. The converse is true of the blood of patients recovering from a hemolytic streptococcal infection, that is, their resistance is due to the *presence* of a substance which is transferable.

Studies in progress indicate that the positive antifibrinolysin tests (resistant plasma-clots) encountered in patients with severe non-streptococcal diseases are frequently like those of newborns in that they are due to absence of a substance—presumably "lytic factor" of Milstone—and not to the presence of streptococcal antifibrinolysin. In other instances both absence of a lytic factor and presence of antifibrinolysin have been demonstrated.

### 13667

## Ultraviolet Sterilizer for Celluloid Tubes.

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In the course of describing a high speed, air-driven centrifuge for the study of viruses, Bauer and Pickels[1] reported the use of celluloid tubes as satisfactory containers capable of withstanding high centrifugal forces. The tubes are transparent, light, flexible, and practically nonbreakable. More recently Horsfall[2] pointed out their advantage over glass containers for the storage of certain labile biological material at the temperature of solid $CO_2$. They now also are used frequently for shipping labile material packed in dry ice.

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[1] Bauer, J. H., and Pickels, E. G., *J. Bact.*, 1936, **31**, 53.

[2] Horsfall, F. L., Jr., *J. Bact.*, 1940, **40**, 559.

The extensive use of celluloid tubes has been handicapped by the difficulty of sterilizing them. Any form of heating deforms them or renders them brittle, and chemical agents such as alcohol are also unsatisfactory. Thus there has existed an urgent need of a practical method of dry sterilization, and the purpose of this paper is to describe a simple apparatus for the sterilization of celluloid tubes by means of ultraviolet light.

The sterilizer consists essentially of a cabinet and 3 enclosed ultraviolet lamps, which, with the aid of a suitable reflector, direct their illumination downward into the openings of the tubes. As celluloid absorbs ultraviolet light, the certainty in sterilization of a tube depends on every element of its internal surface being supplied with an uninterrupted beam of the radiation, either directly from a lamp or by reflection from a good mirror surface. The present apparatus is designed to accomplish this with tubes* which are ½ inch in diameter and 3½ inches in length, and which are provided at the top with impressed threads for metal screw caps. Although such tubes were used in testing the efficacy of the sterilizer, it was found to function equally well, as might be expected, with tubes of other dimensions, provided, however, that the ratio of length to diameter was not greater than 7.

The cabinet is made of wood with an inside width of about 17 inches. Double lapping doors hinged flush with the front side of the cabinet at its ends close into rabbeted recesses which prevent the escape of ultraviolet radiation. It was found to be an advantage to provide the doors with small windows through which the proper operation of the lamps can safely be observed.

The sterilizing radiation is furnished by 3 straight, 30-inch Westinghouse Sterilamp tubes, type 782-30, arranged parallel to each other in a horizontal plane and spaced 2 inches apart, center to center. According to specifications of the manufacturers,† 80% of the radiation generated by these lamps is in the ultraviolet region of 2537 Ångström units. The tubes are made of a special glass which permits a high degree of ultraviolet transmission and consequently they can be furnished at considerably less cost than the more familiar quartz burners. It has been recommended that the tubes be replaced after 4500 hours of intermittent duty since their efficiency decreases slowly after this period. The comparatively high voltage required to operate the lamps can be obtained from either a single transformer or from 3 smaller, individually connected transformers. Supplied by the same manufacturer, these transformers operate on 110 volt, 60 cycle alternating current and consume less than 30 watts for each burner. Very little heat and relatively small amounts of visible light are produced. Heavily insulated wires should be used for electrical conductors between the tubes and the transformers, which can be placed either inside or outside the cabinet. Plain metal clips mounted on strips of insulating material at either end of the cabinet furnish a simple method of holding the lamps and making electrical contact. An ordinary snap or toggle switch outside the cabinet controls the current to the primary side of the transformers.

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* Supplied by the Lusteroid Container Co., Inc., 10 Parker Avenue West, South Orange, New Jersey.

† Westinghouse Electric and Manufacturing Co., 150 Broadway, New York City.

Near the top of the cabinet and centered above the 3 lamps is a metallic reflector made from a 1/16-inch sheet of polished aluminum, commercial grade, measuring 12 inches by 30 inches. It is bent downward along the center line to form an angle of about 140° between the two 6-inch surfaces. The ridge of the reflector is parallel to the central lamp and is situated 4 inches above its center. The reflector may be made of any flat material such as tin-plated iron sheeting which has a high reflectivity in the ultraviolet.

During sterilization the open celluloid tubes are held in test tube racks placed on a shelf whose height is so adjusted that the tops of the tubes are approximately 9 inches below the lamps. It is important that the tubes, especially narrow ones, fit the rack spaces closely enough to be held in an upright position. The limited effective zone for complete sterilization is demarcated on the surface of the shelf by wooden strips 1 inch high which form a fixed rectangular enclosure 5 inches wide and 24 inches long. This enclosure is centered directly beneath the lamps and will accommodate 2 ordinary test tube racks, together holding at least 180 ½-inch tubes.

The efficiency of the sterilizer was determined with several lots of contaminated tubes distributed throughout the effective zone and subjected to the radiation for various lengths of time. The internal wall of each tube, including the threaded section, was contaminated by smearing with a concentrated aqueous suspension of fresh monkey feces which was known to contain large numbers of highly resistant spores. Tests for sterility were performed by filling the tubes with sterile broth, which was allowed to flow from the pipette across the threaded section of the tube. Each tube was incubated at 37°C for 3 days before final reading for sterility or contamination was made. About half of the tubes were usually found to be slightly contaminated after an exposure of 6 hours' duration. Complete sterilization was consistently obtained after an irradiation time of 20 hours. While considerably less time may be sufficient for tubes not purposely and so highly contaminated, the safe practice has been adopted of exposing tubes of any size to the ultraviolet light for 24 hours. The metal screw caps are placed in petri dishes and sterilized separately by any heat. They are then attached with the aid of sterile forceps to the threaded tops of the sterilized tubes, preferably before removing the tubes from the cabinet.

## 13668

## Lymphocytic Choriomeningitis: Two Human Fatalities Following an Unusual Febrile Illness.

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Human infections with the virus of lymphocytic choriomeningitis may assume a number of different clinical forms, *viz.*, aseptic meningitis,[1] meningo-encephalomyelitis,[2] anterior poliomyelitis[3] and grippe.[4, 5] Furthermore, unrecognized infection with this virus appears to be common since many individuals have in their serum neutralizing antibodies for the agent.[5] Nevertheless, acute illness proved to be caused by the virus of choriomeningitis has been reported in less than a hundred individuals and none of these died.[5] Fatalities have been reported among patients assumed to have been infected with this virus;[6] however attempts to isolate the agent from autopsy material were not made, or the results of such attempts were open to question, or the identity of the virus which was recovered was not clearly established. This note is published in order to call attention to a hitherto unrecognized type of severe acute illness associated with the virus of choriomeningitis. A detailed report of the case records of our 2 patients will be given at a later date.

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* Fellow in the Medical Sciences of the National Research Council.

[1] Scott, T. F. McN., and Rivers, T. M., *J. Exp. Med.*, 1936, **63**, 397; Rivers, T. M., and Scott, T. F. McN., *J. Exp. Med.*, 1936, **63**, 415.

[2] Findlay, G. M., Alcock, N. S., and Stern, R. O., *Lancet*, 1936, **1**, 650.

[3] MacCallum, F. O., and Findlay, G. M., *Lancet*, 1939, **1**, 1370.

[4] Lépine, P., Mollaret, P., and Kreis, B., *Compt. rend. Acad. Sci.*, 1937, **204**, 1846.

[5] Armstrong, C., *The Harvey Lectures*, 1940-41, **36**, 39.

[6] Viets, H. R., and Warren, S., *J. A. M. A.*, 1937, **108**, 357; Silcott, W. L., and Neubuerger, K., *Am. J. Med. Sci.*, 1940, **200**, 253; Howard, M. E., *Yale J. Biol. and Med.*, 1940-41, **13**, 161; Machella, T. E., Weinberger, L. M., and Lippincott, S. W., *Am. J. Med. Sci.*, 1939, **197**, 617.

Important clinical and pathological findings in the records of the 2 adult males who died after a severe febrile disease of 12 and 18 days' duration, respectively, may be summarized as follows: The illness of the first patient, a laboratory worker, who had no known contact with the virus of choriomeningitis, was characterized at the onset by fever, malaise, generalized aches, sore throat, vomiting, cough and leukopenia (W.B.C. 2400). Fever rose to 104-105.8°F (by mouth) and continued unabated until death. The white blood cell count rose gradually to 11,350 during the course of the disease; abnormal leucocytes were not encountered in stained smears. A tentative diagnosis of typhoid fever was abandoned when the results of bacteriological studies of blood and stool specimens failed to substantiate it; the terminal clinical diagnosis was "hyperpyrexia of unknown cause." The findings on postmortem examination were those to be expected in a severe febrile disease but the immediate cause of death was not apparent. Patches of bronchopneumonia were present; in a number of areas the usual polymorphonuclear leucocyte exudate was found but in some portions the exudate was composed in part or wholly of mononuclear cells. There was no histopathological evidence of meningitis, choroiditis or encephalitis.

The second individual assisted at the autopsy of the first patient but he sustained no known injury during the procedure. Eight days later he developed malaise and low grade fever; these continued for several days when he was found to have leukopenia (W.B.C. 2500) and mild pharyngitis. The patient was hospitalized because he was thought to have a mild upper respiratory infection in addition to his recognized chronic active pulmonary tuberculosis. He became increasingly ill and died 17 days later. During the course of the disease the pharyngitis increased in intensity and ultimately became necrotizing in character. Hyperpyrexia developed and continued until a day before death. A diffuse erythematous rash appeared for several days. Bleeding from the mucous membranes developed and was accompanied later by hemorrhages into the skin and from the external auditory canal. The white blood cell count rose progressively and terminally it reached 33,000 per cu mm with immature forms constituting 13% of the total. Acute leukemia and "a virus infection" were considered as possible clinical diagnoses at the time of exitus. On postmortem examination numerous hemorrhages were encountered; these included a subdural hematoma and a large clot in one renal pelvis. Extensive necrotizing tonsillitis and pharyngitis were present. Several areas of hemorrhagic consolidation were found in the lungs as well as foci of chronic ulcerative tuberculosis. Histologically the acute pneumonic process was characterized by infiltrations of mononuclear cells in the alveolar septa, by proliferation of alveolar cells and by an exudate consisting of large pale-staining mononuclear cells mixed with red blood cells and fibrin. Microscopic study revealed the presence of loose cuffs of lymphocytes about small vessels in several organs; although not conspicuous the infiltrations were found most often in the periportal spaces of the liver, in the meninges and in the brain substance.

It is of interest that lumbar puncture was not done on either patient. There appeared to be no clinical evidence to warrant such a procedure.

The virus of choriomeningitis was isolated from postmortem blood and from brain tissue of the first patient, and from lung and brain tissue of the second patient. Suspensions of each specimen were inoculated into 2 normal guinea pigs and also, in most instances, into 6 mice. All of the inoculated normal guinea pigs developed fever within a few days and, unless sacrificed for passage material, they subsequently showed the usual signs of severe lymphocytic choriomeningitis[1] and all died. Practically all of the mice injected with human material became sick 7 to 10 days later but about 15% of them ultimately recovered; the remaining mice died in convulsions or were sacrificed when moribund.

The infectious agent recovered from the various tissues of the two patients was identified in each instance as the virus of choriomeningitis. This was done by demonstrating the specific soluble antigen of choriomeningitis[7] in extracts prepared from splenic tissue of guinea pigs of the initial and of each subsequent passage. Furthermore, the agent isolated from each tissue was shown to be incapable of producing obvious disease when inoculated either into guinea pigs or into mice known to be immune to the W.E. strain[1] of the virus of choriomeningitis, while portions of the same tissue suspensions uniformly produced a fatal disease in normal animals. Finally, those mice which survived infection with the newly-isolated strain of virus were later found to resist 1000 M.L.D. of the W.E.

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[7] Smadel, J. E., and Wall, M. J., *J. Bact.*, 1941, **41**, 421.

strain given intracerebrally. It may be mentioned that the histopathological lesions in the central nervous system of guinea pigs and mice that succumbed to infection with the virus isolated from the 2 patients were more severe than those generally encountered in animals infected with the virus of choriomeningitis;[1] not only an extensive choriomeningitis was present but also a true encephalitis.

The occurrence of disease in all guinea pigs and most mice inoculated with the human material clearly indicated that the virus of choriomeningitis had been present in the inocula and that it had not been encountered adventitiously.[†] In order to remove any doubt about this a portion of each tissue was taken from storage at –70°C and after fresh suspensions had been prepared they were again inoculated into normal guinea pigs or mice. The virus was reisolated and reidentified from each specimen.

*Summary.* The virus of lymphocytic choriomeningitis was isolated from the tissues of two patients who died following an undiagnosed acute systemic disease.

### 13669 P

## Morphological Structure of the Virus of Vaccinia

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Elementary bodies of vaccinia which represent the infective unit of the disease[1] are relatively large, *i. e.*, 235-250 mμ[2] in diameter. They respond to osmotic influences,[3] are antigenically complex,[4] and possess certain chemical and biologically active constituents in common with bacteria and mammalian cells.[5] The virus of vaccinia, therefore, might be regarded as a complex entity which more closely resembles bacteria than crystallizable plant viruses, differing from the former principally in respect to size and growth requirements; only living cells are capable of serving as an adequate culture medium, because it appears that the elementary bodies lack certain enzyme systems essential for independent growth and metabolism. In an attempt to detect morphological structure in the elementary bodies of vaccinia, preparations of the virus were examined by means of the RCA electron microscope.[6]

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[†] The virus of lymphocytic choriomeningitis was also isolated by Dr. R. S. Muckenfuss, of the Department of Health, New York City, from tissue of the second patient mentioned in this report.

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[1] Smadel, J. E., Rivers, T. M., and Pickels, E. G., *J. Exp. Med.*, 1939, **70**, 379.

[2] Pickels, E. G., and Smadel, J. E., *J. Exp. Med.*, 1938, **68**, 583.

[3] Smadel, J. E., Pickels, E. G., and Shedlovsky, T., *J. Exp. Med.*, 1938, **68**, 607.

[4] Smadel, J. E., Hoagland, C. L., and Rivers, T. M., *J. Bact.*, 1941, **41**, 57.

[5] (a) Hoagland, C. L., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.*, 1940, **71**, 737; (b) Hoagland, C. L., Ward, S. M., Smadel, J. E., and Rivers, T. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 669; *J. Exp. Med.*, 1940, **72**, 685; *J. Exp. Med.*, 1941, **74**, 69, 133.

The present studies show that elementary bodies of vaccinia possess a definite and characteristic structure. In general their shape is roughly comparable to that of a brick; they are almost rectangular when viewed both from above and from the side. Furthermore, 5 circumscribed areas of increased density are found in the bodies. These appear as dark disks in the micrographs and the arrangement of the 5 is similar to the spots on dice. Prior to our realization of the importance of rewashing stored preparations of virus[1] immediately before examination in the electron microscope different results were obtained. The particles appeared somewhat oval rather than rectangular and were uniformly opaque; these pictures were similar to those obtained previously by others.[7] Apparently under these conditions sufficient protein was adsorbed on the surface of the particles to scatter almost all of the electrons in the electron beam. This protein in stored suspensions of virus probably appears during the course of spontaneous disintegration of a certain number of elementary bodies, since a few ghosts are found in all preparations, and this material is removed from the bodies by washing.

Alkaline treatment of elementary bodies liberates a nucleoprotein antigen from the particles without completely dissolving them.[4] Electron micrographs of films of elementary bodies treated with N/50 NaOH for ½ minute show that certain changes have occurred. Some of the bodies are swollen and show rounded outlines and indistinct edges. Their substance is less dense, resulting in an accentuation of the areas of condensation. Extraction with N/10 NaOH for 10 minutes lakes the virus particles almost completely and even the condensed areas have practically disappeared. Certain of the ghosts formed in this manner present gaps in their surfaces through which in some instances internal substances stream forth.

Control materials, consisting of supernatant fluid from which virus had been sedimented and of particles from normal kidney cells obtained by the method of differential centrifugation identical with that used for the virus of vaccinia, contained no structures which resembled elementary bodies. The present observations indicate that the elementary body of vaccinia has a morphological structure similar in some respects to that of bacteria[7, 8] and different from that of plant viruses.[9]

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[6] Zworykin, V. K., Hillier, J., and Vance, A. W., *Elec. Eng.*, 1941, **60**, 157.

[7] von Borries, B., Ruska, E., and Ruska, H., *Klin. Woch.*, 1938, **17**, 921.

*Summary.* The pictorial data obtained by means of the electron microscope show a remarkable regularity in the morphology of elementary bodies of vaccinia. The virus particles apparently have an internal structure and some sort of limiting membrane.

### 13670 P

## Experiments on Transfer of Cutaneous Sensitivity to Simple Compounds.

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In the course of experiments to passively transfer skin sensitiveness to simple compounds an attempt was made to induce sensitivity by injecting peritoneal exudates from sensitized animals. Guinea pigs were rendered sensitive to picryl chloride in the manner previously described,[1] using conjugates (guinea pig stromata treated with picryl chloride) in conjunction with intraperitoneal injections of killed tubercle bacilli. To produce exudates, killed tubercle bacilli (or tuberculin) were injected intraperitoneally about 3 weeks from the beginning of the treatment, when substantial tuberculin hypersensitivity was established.

On injecting such exudates intraperitoneally into normal animals, the recipients in most of the experiments were seen to develop sensitivity to picryl chloride; when then a drop of an oil solution of the substance was put on the skin, erythematous reactions, mostly of high color, were apparent on the next day. The phenomenon was found to be due not to the clarified fluid but to the sediment obtained upon centrifugation. Among the possible explanations, an active

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[8] (a) Mudd, S., and Lackman, D. B., *J. Bact.*, 1941, **41**, 415; (b) Morton, H. E., and Anderson, T. F., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 277.

[9] (a) Anderson, T. F., and Stanley, W. M., *J. Biol. Chem.*, 1941, **139**, 339; (b) Kausche, G. A., Pfankuch, E., and Ruska, H., *Naturwissensch.*, 1939, **27**, 292.

[1] Landsteiner, K., and Chase, M. W., *J. Exp. Med.*, 1941, **73**, 431.

sensitization through residual antigenic material in the peritoneal exudates seems rather improbable because a transfer was possible also with the exudate of animals in which the injection of dead tubercle bacilli and picryl stromata was made under the skin of the neck (using as a vehicle "Aquaphor" (Duke Laboratories) and paraffin oil, according to a method devised by Freund[2]). This is further supported by the appearance of the sensitivity after a short interval, namely, 2 days after injection of sufficient material, and fading of the reactivity within a few days. Finally, there is preliminary evidence that moderate heating sufficient to kill the exudate cells abolishes the effect. Consequently one would be inclined to assume that the sensitivity is produced by an activity in the recipient of the surviving cells, if not by antibodies carried by these. Positive results were obtained with about 1.5 cc of sediment, which were given in 3 portions on successive days or all at one time; with the present procedure this necessitates the use of quite a number of donors.

Definite results have been obtained with an acylchloride, o-chlorobenzoyl chloride; the majority of experiments have been made with citraconic anhydride, one of the acid anhydrides shown by Jacobs, *et al.*,[3, 4] to produce a new and particular form of reaction (in animals), namely, urticaria-like reactions often with pseudopods appearing immediately after a scratch-test. Positive transfer in human beings had been mentioned briefly by Kern[5] with serum from a patient sensitive to phthalic anhydride, suggesting further experimental study (*vide* Jacobs). We found that sera from highly sensitive guinea pigs which have received intensive treatment with intracutaneous injections of oil solutions of citraconic anhydride contain an antibody which upon intraperitoneal injection into normal guinea pigs render them sensitive. Upon scratch tests made through a drop of a solution of this incitant in dioxane,[3] the recipients gave reactions similar to the immediate ones seen in actively but not highly sensitized guinea pigs. However, upon intracutaneous injection of potent sera (amounts of 0.15 to 0.05 cc were used) into normal albino guinea pigs, intense reactions were elicited within a few minutes in scratch tests made on the prepared site, consisting in swelling and a pinkish coloration, most often with pseudopods and extending over an area up to 3 cm in diameter. The color receded within an hour or so, whereas the swelling persisted for several hours more; the next day the reaction was no longer present. Such reactions occurred in the sensitized sites also when the scratch test was made elsewhere on the normal skin or an oil solution was injected subcutaneously. Furthermore, reactions, even stronger ones (*e. g.*, 5 cm x 4 cm), were obtained at the prepared sites upon subcutaneous injection of a conjugate made with citraconic anhydride and guinea pig serum. The prepared areas were commonly tested after one or two days but are still well reactive after 3 days. Whether the immediate skin reaction described is attributable to an anaphylactic antibody[6, 7] is being investigated. The essential facts are recapitulated in Table I.

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[2] Freund, J., PROC. SOC. EXP. BIOL. AND MED., in press. We are indebted to Dr. Freund for the information.

[3] Jacobs, J. L., Golden, T. S., and Kelley, J. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, **74**.

[4] Jacobs, J. L., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 641.

[5] Kern, R. A., *J. Allergy*, 1939, **10**, 164.

TABLE I.

| Treatment of donor pigs | Material injected for passive trans. | Site of inj. | Method of testing | Type of reaction |
|---|---|---|---|---|
| Picrylated g.p. stromata and killed tb. bacilli i.p. | Peritoneal exudate induced with killed tb. bacilli 3 wks later | i.p. | Oil sol. of picryl chloride on surface of skin | 24 hr—erythematous reaction |
| Same | Supernatant from exudate | i.p. | Same | None |
| Same | 1.5 cc cells from exudate | i.p. | Same | 24 hr—erythematous reaction |
| Same | As above but heated to 48° 15 min. | i.p. | Same | None |
| Prolonged treatment with citraconic anhydride | Serum | i.p. | Scratch test with citraconic anhydride | Immediate urticarial reaction, at times with pseudopods |
| Same | Serum | intracut. | Scratch test locally or at a distance | Immediate urticarial reaction with pseudopods |

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[6] Landsteiner, K., and Jacobs, J., *J. Exp. Med.*, 1936, **64**, 625.

[7] Landsteiner, K., and Chase, M. W., *J. Exp. Med.*, 1937, **66**, 337.

13671

# Red Staining of Paws and Whiskers as in Vitamin $B_2$-Complex Deficient Rats after Dehydration.

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The accumulation of red material on the paws, nose, whiskers and periorbital regions of rats has been attributed to a deficiency of the vitamin G ($B_2$) complex in these animals.[1] Although originally described as blood staining, recent investigations have shown that this dried red stained material consists primarily of porphyrin compounds,[2, 3] although a small amount of blood may be present.

The lesions have been described in rats on both riboflavin-deficient diets[3, 5] and more recently on pantothenic acid-deficient diets.[4, 6] McElroy[2] observed that the red material arose from the Harderian (lacrimal) glands and failed to develop in rats deprived of pantothenic acid if the Harderian glands had been previously removed.

Smith and Sprunt[5] related this phenomenon to dehydration and this observation has been confirmed recently by Figge and Atkinson.[6] The present investigation was designed to determine whether the B-vitamins fed singly or together could alter the course of the development of these lesions resulting from dehydration.

One hundred and three rats were used and grouped as shown in Table I.

In order to impose the same strain on both groups, Purina Dog Chow was available at all times for the rats receiving no water, and water was available constantly for the rats receiving no food. Individuals in both groups began to die at approximately the same time, although the average longevity was slightly greater in the food starved rats.

The synthetic vitamins were given in water solution but the daily dose per rat including those containing the 7 vitamins was always contained in one drop of solution.

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[1] Smith, S. G., PROC. SOC. EXP. BIOL. AND MED., 1932, **30**, 198.

[2] McElroy, L. W., Salomon, K., Figge, F. H. J., and Cowgill, G. R., *Science*, 1941, **94**, 467.

[3] Chick, H., Macrae, T. F., and Worden, A. N., *Biochem. J.*, 1940, **34**, 580.

[4] Unna, K., *J. Nutrition*, 1940, **20**, 565.

[5] Smith, S. G., and Sprunt, D. H., *J. Nutrition*, 1935, **10**, 481.

[6] Figge, F. H. J., and Atkinson, W. B., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 112.

TABLE I.
Incidence and Severity of Red Staining in Dehydrated Rats With and Without Vitamins in Comparison with Food Starved Rats Receiving Water.

| Group | No. of rats | Initial age, avg days | Water regime | Food and vitamin* regime | Average longevity, days | No. showing red staining | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | ++++† | +++ | ++ | + | 0 |
| A | 10 | 82 | No water | Chow | 9 | 1 | 9 | 0 | 0 | 0 |
| B | 16 | — | ” ” | ” | — | 1 | 14 | 1 | 0 | 0 |
| C | 13 | 82 | ” ” | ” + P.A. | 10 | 2 | 10 | 0 | 1 | 0 |
| D | 5 | 125 | ” ” | ” + P.A. | 11 | 1 | 4 | 0 | 0 | 0 |
| E | 10 | 125 | ” ” | ” + R.F. | 11 | 1 | 8 | 1 | 0 | 0 |
| F | 10 | 125 | ” ” | ” + $B_1$, R.F., $B_6$, P.A., N.A., Inos., Cho. | 13+ | 0 | 7 | 3 | 0 | 0 |
| G | 10 | 125 | ” ” | Chow + B.Y. | 13 | 0 | 8 | 2 | 0 | 0 |
| H | 10 | 82 | Water | None | 10 | 0 | 0 | 0 | 0 | 10 |
| I | 14 | — | ” | ” | — | 0 | 0 | 0 | 0 | 14 |
| J | 5 | 125 | ” | ” | 13+ | 0 | 0 | 0 | 0 | 5 |

*Pa.A. = pantothenic acid, 100$\gamma$/rat/day (C); 300$\gamma$/rat/day (D); R.F. = riboflavin, 40$\gamma$/rat/day (E). $B_1$, 13.3$\gamma$; R.F., 16.6$\gamma$; P.A., 66.6$\gamma$; N.A. = nicotinic acid, 66.6$\gamma$; $B_6$, 66.6$\gamma$; Inos. = inositol, 666$\gamma$; Cho. = choline, 666$\gamma$/rat/day (F). B.Y. = brewer's yeast, 0.5 gr./rat/day (G).

†++++ indicates that all 4 sites (nose, whiskers, paws, and eyes) have marked staining as demonstrated by Fig. 1,A. +++, ++ and + are simply graded down from that standard.

In the water-deprived rats food consumption fell off in a short while. This includes, of course, the consumption of brewer's yeast in Group G, which naturally obscures the full effect of this source of natural B-complex vitamins. The group receiving the 7 synthetic vitamins and the brewer's yeast experienced only slightly greater longevity, and the severity of the lesions was modified to only a slight extent.

The sites at which the red porphyrin material accumulates are the paws, nose, whiskers and eyes, as shown by photograph A, Fig. 1. In all the water-deprived rats this accumulation began to appear on the third day and tended to grow progressively worse during the experiment.

Due to weakness before death the food-deprived rats often failed to consume water for 24 to 48 hours before death, resulting in a moist pink staining of paws and nose. In no case, however, were the lesions extensive enough to be confused with the picture presented by the water-deprived rats.

Under the conditions of our experiment the administration of neither riboflavin nor pantothenic acid has any appreciable effect on the lesion. The combination of the synthetic vitamins ($B_1$, $B_6$, riboflavin, pantothenic acid, nicotinic acid, inositol, choline) and the combination of natural vitamins in brewer's yeast had only a minimal effect on the longevity and severity of symptoms.

The characteristic red stained incrustations on nose, whiskers,

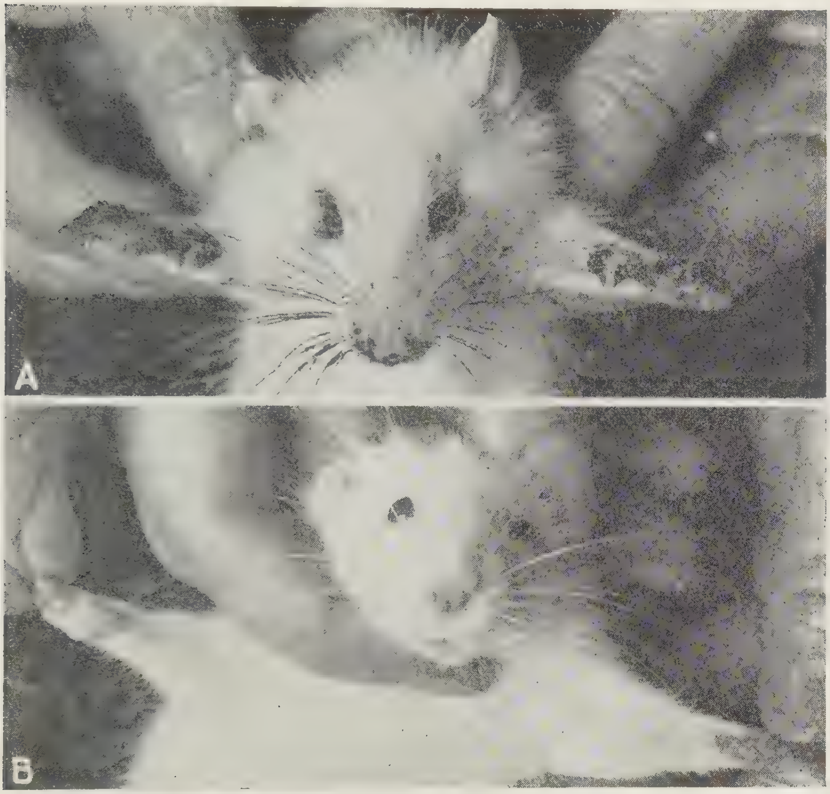

**FIG. 1.**
A. Rat deprived of water for 7 days but receiving food.
B. Rat deprived of food for 7 days but receiving water.

paws and eyes, observed in riboflavin or pantothenic acid deficiencies are very similar, if not identical, to those observed when dehydration is directly induced. Although the vitamins tested under the conditions imposed in these dehydration experiments were negligible in their effectiveness against the characteristic symptoms, it is still probable that these vitamins, particularly pantothenic acid and riboflavin, are concerned with water metabolism.

We wish to thank Merck and Company, Rahway, N.J., for generously supplying us with the vitamin $B_6$ and calcium pantothenate used in this work.

### 13672

## Phytopharmacological Reactions of Blood after Sulfa Compounds.

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Among the commonest reactions following administration of sulfanilamide and its derivatives are skin manifestations of all kinds. The most interesting of these perhaps are such bullous or pemphigoid eruptions as are described by Wien and Lieberthal,[1] Epstein,[2] Loveman and Simon,[3] Bettley,[4] Omens and Robbins,[5] and others. Inasmuch as Macht and Pels some years ago reported a specific and characteristic phytotoxic reaction produced by pemphigus blood sera in these PROCEEDINGS[6] and later in a series of comprehensive papers[7, 8, 9] reporting results obtained with sera of nearly three thousand patients, it was deemed advisable to inquire whether specimens from patients with pemphigoid lesions effected by the sulfa derivatives would exhibit similar phytotoxicity for seedlings of *Lupinus albus.* Accordingly, a series of experiments was made with regard to the influence on plant growth of rabbit and human blood sera drawn after ingestion of sulfa drugs.

*Materials Studied.* The compounds studied in 3 sets of experiments were sulfanilamide, neoprontosil, sulfathiazole, sulfapyridine, sulfadiazine, sulfaguanidine and succinyl sulfathiazole. In the first series 0.5 and 1.0 g doses of the respective drugs were given adult rabbits weighing 2 to 3 kg apiece. Such doses ensured a concentration of these drugs in the blood comparable to that effected in humans receiving full doses of the sulfa compounds, as standard biochemical tests demonstrated. Normal blood specimens were drawn before the drugs were given by stomach tube and others were taken 6 and 24 hours afterward. In the second series, blood sera were obtained from cases treated with various sulfa drugs, and their phytotoxicity was compared with that of normal human blood. In the third series, the effects of various concentrations of the respective chemicals on the growth of seedlings was studied in plant-physiological saline.

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[1] Wien, M. S., and Lieberthal, E. P., *J. A. M. A.*, 1941, **117**, 850.

[2] Epstein, E., *Arch. Dermat. and Syph.*, 1940, **41**, 61.

[3] Loveman, A. B., and Simon, F. A., *Arch. Dermat. and Syph.*, 1939, **40**, 29.

[4] Bettley, F. R., and Simon, P., *Brit. M. J.*, 1939, **1**, 1177.

[5] Omens, D. V., and Robbins, J. B., *Arch. Dermat. and Syph.*, 1939, **40**, 633.

[6] Macht, D. I., and Pels, I. R., PROC. SOC. EXP. BIOL. AND MED., 1927, **25**, 237.

[7] Pels, I. R., and Macht, D. I., *Arch. Dermat. and Syph.*, 1929, **19**, 640.

[8] Pels, I. R., and Macht, D. I., *Arch. Dermat. and Syph.*, 1931, **23**, 601.

[9] Macht, D. I., *Acta Dermatologica*, 1932, **18**, 126.

*Method of Study.* The method employed has been described by the author and associates in numerous publications.[10, 11] The effect of 1% solutions of blood sera was compared with that of plant-physiological saline or Shive solution on growth of roots of *Lupinus albus* for 24 hours in the dark at 20°C. The phytotoxic index of growth for any unknown solution is expressed by the formula, Index = X/N, or average increment of root growth in unknown solution divided by growth of controls under identical conditions. The author has found the phytopharmacological test quite accurate and reliable in experienced hands, and its results have proven valid on statistical analyses.[12] The laboratory findings have been in striking accord with the final clinical diagnoses of physicians submitting blood sera of pemphigus patients for phytopharmacological examination, as exemplified by the recent paper of Gellis and Glass.[13]

*Results.* Thirty experiments each were made with (1) blood sera of rabbits, (2) specimens from patients treated with sulfa compounds, and (3) aqueous solutions of the drugs. It was found that blood sera of rabbits receiving massive doses of the various compounds exhibited phytotoxic readings higher than the index given by normal rabbit serum (70%), as shown in Table I.

The figures obtained with sera from human patients treated with sulfanilamide and derivatives are given in Table II. Here again, the figures shown are definitely higher than those obtained with normal human blood serum, the index of which is from 70 to 75%. Experiments made with aqueous or plant-physiological solutions of the

TABLE I.
Effect on *Lupinus* Seedlings of 1% Solutions of Sera of Rabbits Receiving 0.5 g of Respective Sulfa Compounds.

| Drug administered | Phytotoxic index % |
|---|---|
| Sulfanilamide | 79 |
| Sulfapyridine | 86 |
| Sulfathiazole | 81 |
| Sulfadiazine | 83 |
| Sulfaguanidine | 79 |
| Succinyl sulfathiazole | 81 |
| Neoprontosil | 96 |

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[10] Macht, D. I., and Livingston, M. B., *J. Gen. Physiol.*, 1922, **4**, 573.

[11] Macht, D. I., *Protoplasma*, 1937, **27**, 1.

[12] Macht, D. I., and Macht, M. B., *J. Lab. and Clin. Med.*, 1941, **26**, 597.

[13] Gellis, S., and Glass, F. A., *Arch. Dermat. and Syph.*, 1941, **44**, 321.

TABLE II.
Effect on *Lupinus* Seedlings of 1% Solutions of Sera of Patients to Whom Respective Sulfa Compounds Had Been Given.

| Drug administered | Phytotoxic index % |
|---|---|
| Sulfanilamide | 76 |
| Sulfapyridine | 94 |
| Sulfathiazole | 77 |
| Sulfadiazine | 85 |
| Sulfaguanidine | 83 |
| Succinyl sulfathiazole | 78 |

sulfa compounds in concentration of 1:10,000 on growth of *Lupinus albus* seedlings also yielded interesting results. None of the drugs examined was very toxic for root growth, the phytotoxic indices in concentrations of 1:10,000 ranging from 80 to 90%. Of greater interest were the results obtained with more dilute solutions of the sulfa compounds. When low concentrations of from 1:100,000 to 1:250,000 were employed, a stimulation of root growth was noted. Complete data concerning these plant-physiological experiments will be published elsewhere.

From the standpoint of clinical medicine the above findings with blood sera are significant in that they designate the phytopharmacological test as a convenient means of differentiating true pemphigus from pemphigoid dermatoses effected by sulfanilamide and its derivatives. Plant-physiological experiments with saline solutions of these drugs are of interest in connection with the mechanism of action of sulfa compounds, which is still not completely understood.

*Summary.* (1) The Macht-Pels phytotoxic test for pemphigus was applied to sera of rabbits and human beings treated with sulfanilamide and 6 derivatives. (2) None of these sera exhibited a greater toxicity than normal blood for seedlings of *Lupinus albus*. (3) The phytopharmacological test thus affords a convenient means of differentiating between true pemphigus and the pemphigoid eruptions produced by sulfa drugs. (4) Hydroponic experiments on *Lupinus albus* seedlings placed in solutions of seven sulfa derivatives revealed a diphasic effect on growth of the roots. Low concentrations stimulated while higher concentrations inhibited root growth.

13673

# Action of Ascorbic Acid and Hydrogen Peroxide on Collagen and Thymonucleohistone.*†

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A degradation of mucins and polysaccharides by ascorbic acid or other enediols and hydrogen peroxide, which consisted of a loss of viscosity and a reduction in particle size of the substrate, was reported recently.[1] We have further observed that these same agents cause a similar irreversible reduction of viscosity of solutions of two other biologically significant substances, collagen and thymonucleohistone. The changes which occur in collagen, so-called "depolymerization," are of special interest in the light of Wolbach's work demonstrating the necessity of ascorbic acid for the formation of collagen fibers *in vivo*.[2]

## *Experimental.*

*Collagen.* Collagen was prepared by extracting the tendons of rats' tails according to the method of Bessey.[3] The resulting solution is water clear and extremely viscous. Upon dialysis, it forms a firm gel in the dialyzing sack. Collagen can be precipitated from such an extract as a filamentous precipitate by the addition of an equal volume of 2.3 M potassium phosphate, pH 6.3.

The degradation of collagen by ascorbic acid and hydrogen peroxide was illustrated by the following experiment. One ml of M/5 phosphate buffer containing 20 mg of ascorbic acid and 1 ml of 1% hydrogen peroxide were added to 50 ml of collagen extract. The control solution contained collagen, phosphate buffer and hydrogen peroxide. The pH of both solutions was 4.2. The temperature was kept at 6° to inhibit spontaneous degradation of collagen. The changes in relative viscosity which occurred are shown below.

| | | Immediately after mixing | Sixteen hrs later |
|---|---|---|---|
| A | Collagen + ascorbic acid + hydrogen peroxide | 8.1 | 2.1 |
| B | Control | 8.1 | 7.0 |

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* This is publication No. 58 of the Robert W. Lovett Memorial for the study of crippling disease, Harvard Medical School, Boston, Massachusetts.

† The expenses of this investigation have been defrayed by a grant from the Commonwealth Fund.

[1] Robertson, W. v. B., Ropes, M. W., and Bauer, W., *Biochem. J.*, 1941, **35**, 903.

[2] Wolbach, S. B., *Am. J. Path.*, 1933, **9**, 689.

[3] Bessey, O. C., personal communication.

Solution B yielded a typical precipitate with 2.3 M phosphate buffer, whereas solution A gave none. The irreversibility of this degradation of collagen was shown by the fact that upon dialysis solution B formed a firm gel, whereas solution A exhibited further decrease in relative viscosity to 1.7.

*Thymonucleohistone.* Minced thymus gland was extracted twice with 10% sodium chloride at 4° for 2 days. Following centrifugation the viscous solution was precipitated by pouring into 3 volumes of 1% acetic acid. The resulting fibrous precipitate was redissolved in M/5 phosphate buffer, pH 6.2, containing 5% NaCl.

The addition of 2 ml of M/5 phosphate buffer containing 20 mg of ascorbic acid and 1 ml of 5% hydrogen peroxide to 25 ml of this solution caused a breakdown of the nucleohistone within one hour as indicated by the flocculent precipitate obtained in 1% acetic acid. The control solution, lacking ascorbic acid, yielded a fibrous, ropey precipitate even at the end of 24 hours.[‡] The relative viscosity of the degraded nucleohistone after one hour was 1.28 as compared with 4.51 for the control solution.

*Summary.* Ascorbic acid and hydrogen peroxide cause an irreversible reduction of viscosity of solutions of collagen and thymonucleohistone.

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## 13674

## An Unsuccessful Search for Dopa in Protein Hydrolysates.

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Dopa (*l*(—)-3,4-dihydroxyphenylalanine) occurs in free form in the broad bean *(Vicia faba)*[1] in the Georgia velvet bean *(Stizolobium deeringianum)*,[2] and in certain insects.[3] It has not been isolated from protein hydrolysates. Dunn[4] has suggested that its ease of oxidation might account for its apparent absence from hydrolysates.

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[‡] The change in the precipitate was similar to that observed in the case of synovial fluid mucin, which was illustrated in a previous publication.[4]

[4] Robertson, W. v. B., Ropes, M. W., and Bauer, W., *J. Biol. Chem.*, 1940, **133**, 261.

[1] Torquati, T., *Arch. farmocol. sper.*, 1913, **15**, 213, 308; Guggenheim, M., *Z. physiol. Chem.*, 1913, **88**, 276.

[2] Miller, E. R., *J. Biol. Chem.*, 1920, **44**, 481.

[3] Schmalfuss, H., and Muller, A., *Biochem. Z.*, 1927, **183**, 362.

In an orienting experiment, 45 mg of dopa were boiled under reflux with constant boiling HCl solution. At the end of 20 hours, 75% of the dopa still was present; at the end of 238 hours, 27% remained. The analytical method employed has been described elsewhere.[5]

Several proteins (purified egg albumin, casein, edestin, fibrin) were hydrolyzed with sodium hydroxide, sulfuric acid, or hydrochloric acid. No dopa could be detected in any of the hydrolysates. When dopa equivalent to 1% of the protein was added prior to hydrolysis, no dopa could be found in the alkaline hydrolysates, but 70 to 90% of the added material was found in the acid hydrolysates. Since a part of the loss resulted from adsorption of the dopa on the charcoal used for clarification of the hydrolysates, it is probable that only slight destruction occurs when the compound is boiled with acid in the presence of protein.

Since dopa is known to occur in the Georgia velvet bean, the protein of this bean was investigated. The beans were powdered in a drug mill, and the powder was thoroughly extracted with 20% trichloracetic acid until the washings no longer gave a test for dopa. The insoluble residue then was washed with 80% alcohol until the washings no longer were acid to litmus. The dopa-free protein was hydrolyzed with HCl. No dopa could be detected in the hydrolysate, although positive tests were obtained when dopa was added to the protein prior to hydrolysis.

Since some samples of charcoal were found to adsorb dopa quantitatively, hydrolysates of the velvet bean protein were clarified with cuprous oxide.[6] Again, tests for dopa were negative unless the substance had been added to the mixture either before or after hydrolysis.

When the velvet bean protein hydrolysate was adjusted to neutrality and extracted with butyl alcohol,[7] any dopa present was found to be in the proline fraction—*i. e.*, in solution in the butyl alcohol-water mixture present in the flask from which the butyl alcohol was distilled. No dopa was found in this fraction unless it had been added to the protein or to the hydrolysate.

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[4] Dunn, M. S., in Schmidt, C. L. A., *Chemistry of the Amino Acids and Proteins*, Charles C. Thomas, Springfield, 1938.

[5] Arnow, L. E., *J. Biol. Chem.*, 1937, **118**, 531.

[6] Abderhalden, E., and Fuchs, D., *Z. physiol. Chem.*, 1908, **57**, 339.

[7] Dakin, H. D., *Biochem. J.*, 1918, **12**, 1290.

*Comment.* The evidence here presented can be interpreted to indicate that: (1) Dopa is not a constituent of any of the proteins examined. (2) Or, that dopa in combined form is more readily destroyed than is free dopa. (3) Or, that linkages involving dopa are not cleaved by the procedures used for hydrolysis. This interpretation involves the unlikely assumption that combined dopa does not yield a color with the nitrite-molybdate reagent.

*Summary.* Dopa is destroyed rapidly in boiling alkaline solutions, and much more slowly in boiling acid solutions. When dopa was added to proteins (egg albumin, casein, edestin, fibrin, velvet bean protein) prior to acid hydrolysis, most of it could be detected in the hydrolysates. However, no dopa was found in any of the hydrolysates prepared without previous addition of this compound.

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13675

## Ultraviolet Absorption Spectrum of Bovine Phosphatase in Presence and Absence of Added Substrate.

IRWIN W. SIZER. (Introduced by F. O. Schmitt.)

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The ultraviolet absorption spectra of enzymes which have been studied (pepsin,[1] papain,[2] urease,[3] yellow enzymes,[4] milk flavoprotein,[5] ribonuclease[6]) resemble closely those which have been reported for proteins. The absorption maximum at 270-280 mμ and minimum at 240-250 mμ have been attributed to the presence of tyrosine, tryptophane and, to a lesser degree, phenylalanine in the protein molecule. The so-called "end absorption" is ascribed to the presence in the protein of many different amino acids which strongly absorb ultraviolet below a wavelength of about 240 mμ.

The most generally accepted theory of enzyme catalysis postulates the formation of an intermediary enzyme-substrate complex. Spectroscopic evidence for the existence of such a complex is lacking except for the heme containing enzymes, catalase (for discussion *cf.* Green[7]) and peroxidase[8] where the addition of substrate to the enzyme causes a shift of absorption bands in the visible region of the spectrum. The present work presents a study of the ultraviolet absorption spectrum of bovine phosphatase, and the effects of its substrate, sodium $\beta$-glycerophosphate, upon this spectrum.

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[1] Gates, F. L., *J. Gen. Physiol.*, 1934, **18**, 265.

[2] Darby, H. H., *J. Biol. Chem.*, 1941, **139**, 721.

[3] Kubowitz, F., and Haas, E., *Biochem. Z.*, 1933, **257**, 337.

[4] Theorell, H., *Biochem. Z.*, 1935, **278**, 279.

[5] Corran, H. S., Dewan, J. G., Gordon, A. H., and Green, D. E., *Biochem. J.*, 1939, **33**, 1694.

[6] Uber, F. M., and Ells, V. R., *J. Biol. Chem.*, 1941, **141**, 229.

*Methods.* The absorption spectra were taken with a Hilger quartz spectrograph (Model E484) equipped with a Spekker photometer using a tungsten spark. The photographic plates were projected and the match points at the different wave-lengths read by eye. Several bovine phosphatase preparations of different degrees of purity were used. The intestinal phosphatase (prepared by Dr. Gerhardt Schmidt by tryptic digestion of the intestinal mucosa, followed by extraction of lipids and differential salt precipitation of the protein components) was highly purified and contained only the alkaline phosphatase, while the kidney phosphatase, prepared according to Albers,[9] contained both alkaline and acid phosphatase. The enzyme was dissolved in glass distilled water and placed in a 2 cm quartz cell. The duplicate cell contained only the solvent. The pH of the solution was measured with the glass electrode at room temperature immediately after the absorption spectrum had been taken.

*Results.* From the ultraviolet absorption spectrum it is apparent that intestinal phosphatase does not absorb in the visible region,[10] but absorbs strongly in the ultraviolet with a sharp maximum at 278 mμ, a minimum at 253 mμ, and "end absorption" below 236 mμ (Fig. 1, curve A). The absorption spectrum is the same before and after purification of the phosphatase by treatment with $Al(OH)_3$. Essentially similar curves were obtained with several preparations of kidney phosphatase of different degrees of purity, with an average maximum at 275 mμ and minimum at 257 mμ. The small differences in the values for the intestinal and kidney phosphatase can probably be ascribed to the presence of impurities in the kidney phosphatase.

Different proteins have characteristic ultraviolet absorption spectra,[11] and of these the absorption spectrum of phosphatase

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[7] Green, D. E., *Mechanisms of Biological Oxidations*, Cambridge, 1940.

[8] Chance, B., *J. Biol. Chem.*, 1941, **140**, XXIV.

[9] Albers, D., *Biochem. Z.*, 1940, **306**, 143.

[10] Kraemer, E. O., Weil, L., Lanigar, E. B., and Allen, M. T., *J. Frank. Inst.*, 1941, **232**, 587.

[11] Ellinger, F., *Absorptions Spektroskopie im Ultraviolett*, The Hague, 1927.

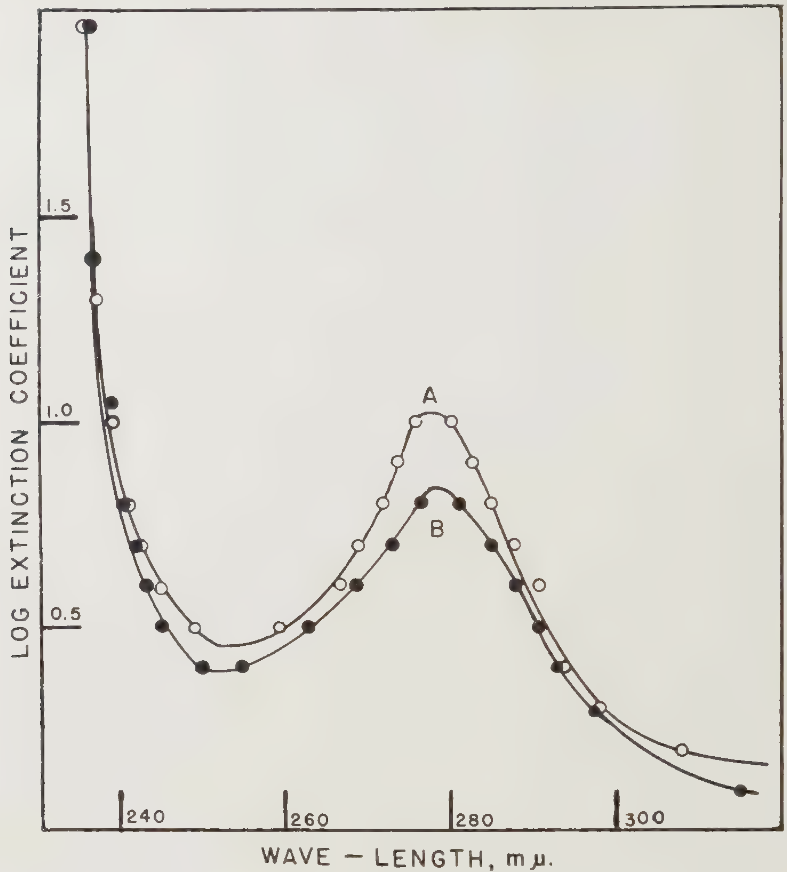

FIG. 1.

Ultraviolet absorption spectrum of 0.009% bovine intestinal phosphatase.
Curve A. No added substrate, pH 4.97.
Curve B. Solution contains 0.1% sodium β glycerophosphate, pH 7.72.

closely resembles that of typical globulins. The suggestion that this enzyme may be a globulin is confirmed by chemical and physical investigations of other workers.[9, 10, 12] An attempt was also made to relate the ultraviolet absorption spectrum to the spectrum of amino acids which might be present in the phosphatase molecule. Of the various amino acids which absorb strongly in the ultraviolet it was found that tyrosine (aqueous solution, 0.05 mg/ml, pH 5.63) has a maximum absorption at 276 mμ and a minimum at 245 mμ, while tryptophane (aqueous solution, 0.015 mg/ml, pH 5.21) has a maximum absorption at 280 mμ and a minimum at 242 mμ. These values agree well with those reported previously[13] and suggest that

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[12] Perlmann, G. E., and Ferry, R. M., *J. Biol. Chem.*, 1942, **142**, 513.

[13] Loofbourow, J. R., *Rev. Mod. Physics*, 1940, **12**, 267.

the ultraviolet absorption spectrum of these 2 amino acids largely accounts for the characteristic absorption spectrum of phosphatase.

A change in ultraviolet absorption would occur on the addition of substrate to enzyme, if a complex is formed of which the absorbing characteristics (arising from increased or decreased saturation, substitution of unsaturated linkages, etc.) are different from the original enzyme and substrate. The effect of substrate on the ultraviolet absorption spectrum of phosphatase was studied by dissolving the enzyme in 0.1% sodium $\beta$-glycerophosphate (Merck). The quartz cell for solvent contained the same concentration of substrate but no enzyme. The absorption spectrum is shown in Fig. 1, curve B. It is apparent from a comparison of the two curves that although the total absorption is less in curve B (possibly due to a difference in pH or concentration of the two solutions), there is no shift in the absorption curve indicative of the formation of an enzyme-substrate complex. As a very sensitive method of detecting any spectral shift of the enzyme caused by the substrate, one beam from the tungsten spark is passed through a solution containing phosphatase plus sodium $\beta$-glycerophosphate in the same quartz cell, while the other beam is passed through two cells, one containing enzyme, the other substrate. Any difference in the absorption of light by these 2 systems must be caused by the formation of an enzyme-substrate complex in the cell containing both enzyme and substrate. Experiments of this type were performed, using both intestinal and kidney phosphatase, but revealed no evidence of an enzyme-substrate complex. The lack of shift in spectrum does not eliminate the possibility that an enzyme substrate complex is formed, but if such a complex exists the manner of combination must be such as to have no appreciable influence on the ultraviolet absorption.

The author is very grateful to Dr. Gerhardt Schmidt, Tufts Medical School, and to Mr. Herbert Jaffe, M.I.T., for samples of phosphatase; and to Professor John R. Loofbourow, M.I.T., for generous assistance and advice in this investigation.

*Summary.* The ultraviolet absorption spectrum of bovine intestinal and kidney phosphatase indicates a maximum at 278 mμ, a minimum at 253 mμ and "end absorption" below 236 mμ. The absorption curve resembles that of typical proteins and suggests that phosphatase is a globulin. Tyrosin and tryptophane in the phosphatase may largely account for its characteristic absorption. No spectroscopic evidence was found for the formation of an enzyme-substrate complex when sodium $\beta$-glycerophosphate was added to a phosphatase solution.

## 13676

# Rôle of Thyroid in Cyclopropane-Adrenalin Tachycardia.*

J. W. STUTZMAN AND W. J. MEEK.

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Cyclopropane greatly enhances the activity of adrenalin on the automatic tissues of the dog's heart. A dose of .01 mg of adrenalin per kilogram intravenously causes only escape phenomena in the unanesthetized animal, while under deep cyclopropane anesthesia this same dose results in multifocal ventricular tachycardia.[1] Decerebration and crossed circulation experiments indicate that the cardiac response to the direct adrenalin stimulation is dependent upon the functional integrity of a brain center above the pons, the heart having been sensitized by cyclopropane acting directly upon it.

Since the thyroid gland appears to sensitize the whole or part of the sympathetic nervous system,[2] the influence of the thyroid on cyclopropane-adrenalin tachycardia has been investigated.

*Methods.* Sixteen dogs were anesthetized with cyclopropane without premedication, intubated to insure an open airway, and connected through a soda-lime carbon dioxide absorber to a 100 liter rubber bag containing a 30 to 32% mixture of cyclopropane in oxygen. This concentration of the anesthetic mixture produces deep surgical anesthesia with at least partial intercostal paralysis in dogs. The animals were allowed to equilibrate on this constant mixture for 30 minutes. The test dose of .01 mg of adrenalin per kilogram in 5 cc of normal saline was then injected into the left radial vein at a constant rate over a 50-second period. The duration of the resulting ventricular tachycardia was determined by observation of the beam of the electrocardiograph and by electrocardiograms (lead II) taken over a 5-minute period beginning with the injection.

Eleven of the animals were then thyroidectomized, care being taken to preserve the superior pair of parathyroid glands. Twenty-six days later the test dose of adrenalin was again injected under cyclopropane anesthesia and the duration of ventricular tachycardia noted.

To determine the effects of thyroid feeding, 9 of the 11 thyroidecto-

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* Made possible in part by a grant from the Wisconsin Alumni Research Foundation.

[1] Allen, C. R., Stutzman, J. W., and Meek, W. J., *Anesthesiology*, 1940, **1**, 158.

[2] Wolf, W., *Endocrinology in Modern Practice*, 2nd ed., W. B. Saunders, 1939.

mized dogs and the 5 intact dogs were given desiccated thyroid glands (Parke, Davis) for 12 days. The dose for 12 of the animals was 400 mg per kilogram per day and for the other 2, 200 mg per kilogram per day for the first 7 days and then 400 mg per kilogram per day for the remaining 5 days. On the thirteenth day the duration of ventricular tachycardia was again determined for all 14 animals.

As a measure of the degree of hypo- and hyperthyroidism, oxygen consumption values were determined on 6 of the dogs by the method of Kunde.[3]

*Results.* The response to the injection of adrenalin during cyclopropane anesthesia before and after thyroidectomy is shown in Table I. In 5 of 11 animals cyclopropane-adrenalin tachycardia was entirely abolished after removal of the thyroids, and in the remaining 6 the duration of the arrhythmia was reduced from an average of 56 to 30 seconds.

That the removal of the thyroid was the essential factor in this reduction was shown by feeding 9 of the dogs desiccated thyroid. Cyclopropane-adrenalin tachycardia reappeared in 3 of the animals and in the others increased in duration.

Following operation there was little residual thyroid tissue, as can be seen from the average decrease in oxygen consumption of 33% for the dogs tested. These results and those obtained by substitution therapy are summarized in Table II.

TABLE I.

Effect of Thyroidectomy and Subsequent Thyroid Feeding on Duration of Cyclopropane-Adrenalin Tachycardia in Dogs.

| | Duration of ventricular tachycardia | | |
|---|---|---|---|
| Dog No. | Intact control sec | Thyroidectomized sec | Thyroid feeding (after thyroidectomy) sec |
| 1 | 46 | 20 | 85 |
| 2 | 35 | 0 | 83 |
| 3 | 67 | 50 | 76 |
| 4 | 40 | 25 | 97 |
| 5 | 45 | 0 | 64 |
| 6 | 55 | 0 | 88 |
| 7 | 68 | 30 | 110 |
| 8 | 52 | 28 | 62 |
| 9 | 64 | 26 | 82 |
| 10 | 51 | 0 | |
| 11 | 48 | 0 | |
| Avg | 52 | 16 | 83 |

Note. The dose of thyroid for dogs 1 to 7 was 400 mg/kg/day for 12 days and for dogs 8 and 9 200 mg/kg/day for the first 7 days and then 400 mg/kg/day for the remaining 5 days.

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[3] Kunde, M. M., and Steinhaus, A., *Am. J. Physiol.*, 1926, **78**, 127.

TABLE II.
Effect of Thyroidectomy and Subsequent Thyroid Feeding on Oxygen Consumption in Dogs.

| | | Thyroidectomized | | Thyroid feeding (after thyroidectomy) | | |
|---|---|---|---|---|---|---|
| Dog No. | Control cc $O_2/M^2/min.$ | cc $O_2/M^2/min.$ | % deviation from control | cc $O_2/M^2/min.$ | % deviation from control | % deviation from hypothyroid level |
| 1 | 129 | 63 | —51 | 171 | +33 | +171 |
| 2 | 115 | 72 | —37 | 197 | +71 | +174 |
| 3 | 106 | 79 | —25 | 158 | +53 | +100 |
| 4 | 156 | 103 | —34 | 235 | +51 | +128 |
| 5 | 139 | 102 | —27 | 236 | +70 | +131 |
| 6 | 108 | 83 | —23 | 186 | +72 | +124 |
| Avg | 125 cc/$M^2$/min. | 84 cc/$M^2$/min. | —33% | 197 cc/$M^2$/min. | +58% | +135% |

Note. The dose of thyroid was 400 mg/kg/day for 12 days.

Surface area calculated by the formula of Meehs (S.A. $= \sqrt[3]{\text{weight}^2} \times 0.112$).

The effect of thyroid feeding in 5 normal dogs was next determined. The dose was 400 mg per kilogram daily for 12 days. When the animals were anesthetized with cyclopropane and the test dose of adrenalin injected on the thirteenth day, one died of ventricular fibrillation. The average duration of ventricular tachycardia for the remaining 4 dogs was 61 seconds, compared to an average of 40 seconds in the same animals before thyroid feeding. Loeb, Bassett, and Friedman[4] reported that regressive changes in the thyroid glands of guinea pigs followed the administration of thyroid substance. It is then possible that in the present work the feeding of thyroid tissue to intact dogs diminished their own thyroid activity, and therefore the total thyroid effect was actually diminished.

*Discussion.* Garrelon and Pascalis[5] believe that adrenalin-chloroform syncope was induced by adrenalin stimulation of the vagus. This resulted in the elimination of a thyroid hormone which was the sensitizing agent. The heart being suddenly sensitized to chloroform, the resulting fibrillation was really a chloroform syncope. The evidence for a primary production of increased tonus in the vagus by adrenalin is not particularly convincing.

Our experiments show that the heart becomes more sensitive to adrenalin and cyclopropane as the thyroid activity is increased. Cyclopropane-adrenalin tachycardia may occur, however, in thyroidectomized animals which show a marked decrease in their basal metabolism, so the thyroid is not absolutely necessary for the reaction. However, it does modify the response.

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[4] Loeb, L., Bassett, R. B., and Friedman, H., PROC. SOC. EXP. BIOL. AND MED., 1930, **28**, 209.

[5] Garrelon, L., and Pascalis, G., *Press. Medicale,* 1930, **1**, 649.

A more plausible explanation for the increase in cyclopropane-adrenalin tachycardia in the hyperthyroid state and the decrease in the hypothyroid state seems to be that the thyroid hormone sensitizes the heart to adrenalin. A cardiac accelerator response to adrenalin greater than the control has been demonstrated when cats,[6] rabbits,[7] and terrapins[8] are in the hyperthyroid state. Thyrotoxic rabbit and dog hearts were reported as more susceptible to irregularities than normal controls.[9] An increased cardiovascular reaction to adrenalin is the basis for a clinical test for hyperthyroidism.[10]

Aumann and Youmans[11] recently demonstrated that the thyroid hormone sensitizes the excitatory adrenergic but not the inhibitory adrenergic neuro-effector division of the autonomic nervous system. The present work on cyclopropane-adrenalin tachycardia also seems to indicate that the thyroid sensitizes the excitatory adrenergic neuro-effector system.

*Summary.* Following thyroidectomy the duration of cyclopropane-adrenalin tachycardia was decreased in the dog. In the hyperthyroid state the period of tachycardia was longer than in the control.

We wish to thank Drs. C. R. Allen and O. S. Orth for assistance with some of the experiments.

## 13677 P

## Excretion of Estrogen in Bile.

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There is considerable evidence that the liver plays an important part in the metabolism of estrogens. This evidence may be summarized as follows: (a) incubation of liver slices with estrogens *in vitro* results in marked diminution in estrogenic activity;[1-7] (b) extraction of minced tissues after injection of estrogens results in recovery of only a small proportion of the quantity injected;[8, 9] (c) little or no estrogenic effect can be detected when ovarian tissue or estrogen pellets are implanted in the portal field;[10-13] (d) high levels of urinary excretion of free estrogen have been reported in patients with cirrhosis of the liver;[14] (e) the hypothesis of inactivation of estrogens by the liver has been supported by studies of the effects of endogenous and exogenous estrogens in normal animals and in animals with liver damage induced by hepatotoxic agents.[6, 15-17]

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[6] Sawyer, M. E., and Brown, M. G., *Am. J. Physiol.*, 1935, **110**, 620.

[7] McDonald, C. H., Shepeard, W. L., Gree, M. F., and DeGroat, A. F., *Am. J. Physiol.*, 1935, **112**, 227.

[8] Lewis, J. K., and McEachern, D., *Bull. Johns Hopkins Hosp.*, 1931, **48**, 228.

[9] Rosenblum, H., Hahn, R. G., and Levine, S. A., *Arch. Int. Med.*, 1933, **51**, 279.

[10] Goetsch, E., *Penn. Med. J.*, 1920, **23**, 431.

[11] Aumann, K. W., and Youmans, W. B., *Am. J. Physiol.*, 1940, **131**, 394.

* J. Ewing Mears Fellow in Medicine and Physiology.

The chemical similarity between natural estrogens and bile acids and the fact that the latter, like the former, disappear rapidly from the blood and urine following intravenous injection in normal animals, but less rapidly in the presence of liver damage, suggested the possibility that estrogens, like bile acids, may be removed from the blood by the liver and subsequently undergo an enterohepatic circulation. The presence of estrogen in the bile after administration of estrone, estradiol and diethylstilbestrol has been reported by Stamler[18] and Dingemanse and Tyslowitz.[19]

Longwell and McKee,[20] employing bile-fistula dogs, found a maximum 72-hour excretion of 8% of the estrogenic activity of 1 mg of subcutaneously injected estrone, practically all of this being in the form of non-ketone estrogen, presumably estradiol. The

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[1] Silberstein, F., Engel, P., and Molnar, K., *Klin. Wchnschr.*, 1933, **12**, 1693.

[2] Zondek, B., *Skand. Arch. Physiol.*, 1934, **70**, 133.

[3] Israel, S. L., Meranze, D. R., and Johnston, C. G., *Am. J. Med. Sci.*, 1937, **194**, 835.

[4] Heller, C. G., Heller, E. J., and Sevringhaus, E. L., *Am. J. Physiol.*, 1939, **126**, P530.

[5] Heller, C. G., *Endocrinology*, 1940, **26**, 619.

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[10] Golden, J. B., and Sevringhaus, E. L., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 361.

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[13] Biskind, G. R., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 266.

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[19] Dingemanse, E., and Tyslowitz, R., *Endocrinology*, 1941, **28**, 450.

[20] Longwell, B. B., and McKee, F. S., *J. Biol. Chem.*, 1942, **142**, 757.

marked discrepancy between their findings and those reported here may be due to the relatively small dose employed by them and to the relatively slow absorption following subcutaneous injection of estrone in oil.

*Experimental Observations.* Studies were made of the excretion of estrogen in the bile of 2 cholecystectomized, bile-fistula female dogs, the bile draining externally into a balloon. Estrogen activity was determined by the biological assay method of Fluhmann,[21] against estrone as a standard. Conjugated estrogen was determined by preliminary boiling with HCl in a water bath at pH 1.0.

No estrogen activity was present in 24-hour specimens of bile obtained from untreated animals. The following findings were obtained following a single intravenous injection of 250,000 I.U. of estrone in 1 cc of absolute alcohol, none of the bile being refed: first 24 hours, free estrogen 100,000 I.U., total estrogen 120,000 I.U.; second 24 hours, free estrogen 88,000 I.U.; total estrogen 120,000 I.U. In another animal, following a single intravenous injection of 250,000 I.U. of estrone, aliquots of bile were taken for analysis daily for 3 days, about 90% being refed by stomach tube. The following results were obtained: first 24 hours, free estrogen 100,000 I.U.; second 24 hours, free estrogen 66,000 I.U.; third 24 hours, free estrogen 66,000 I. U. We have found, as have other observers, that the level of estrogenic activity in the blood after 3-4 hours is only about 10% of that at 10 minutes after intravenous injection, practically none being present after 24 hours. About 10% of the injected estrogen can be recovered from the urine during the first 24 hours, the quantity diminishing during the next 3 days.

The data presented here suggest that there is an enterohepatic circulation of estrogens, similar to that of bile acids. The fact that 34-48% of the quantity of estrogen administered can be recovered from the bile during the second 24 hours, a similar amount having been removed in the bile during the first 24 hours, indicates that the liver can store relatively large quantities of estrogen for at least this period of time.

*Summary.* Studies of the excretion of estrogen in the bile of bile-fistula dogs indicate that extremely large quantities are excreted by this route for at least 3 days following a single intravenous injection of estrone. Viewed in the light of the rapid disappearance of estrogen from the blood and its minimal excretion in the urine under such circumstances, this observation suggests the existence of an enterohepatic circulation of estrogens, similar to that of bile

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[21] Fluhmann, C. F., *Endocrinology*, 1934, **18**, 705.

acids. It throws some doubt upon the hypothesis of *rapid* destruction or inactivation of estrogens by the liver in the intact animal. The liver appears to be capable of storing relatively large quantities of estrogen for at least 24 hours after administration of a single dose of estrone intravenously.

### 13678

## Influence of Estrone upon Formation of Heterophil Antibodies.

E. VON HAAM AND IRENE ROSENFELD.

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In a previous communication[1] we reported on the effect of the female sex hormone upon the formation of antibodies in rabbits immunized against Type I pneumococcus. Administration of estrone totaling 22 and 38 mg given before and during the process of immunization, produced a remarkable increase in specific agglutinating and protecting antibodies. The hormone alone did not elicit any specific antibody-response. Since it is known that Type I pneumococcus contains Forssman antigen (Powell and Jamieson[2]), it seemed of interest to investigate the effect of hormone-administration upon the production of heterophil antibodies, following the work of Levine, Bullowa, and Katzin.[3]

*Material and Methods.* Our above-mentioned sera were tested against 2% suspensions of washed human blood cells of groups AB, A, B, and O, and sheep erythrocytes. Serial dilutions of each serum were made in amounts of 0.15 cc beginning with a dilution of 1:2.5. To each diluted serum 0.1 cc of a 2% suspension of erythrocytes was added. The mixtures were shaken for 1 minute, incubated at 37°C for 2 hours and read macroscopically.

*Results.* Agglutinins for group A erythrocytes were demonstrable in the sera of the control animals up to a dilution of 1:2.5; for group AB, in 3 out of 6 animals up to a dilution of 1:5; for group B in the serum of only 1 rabbit to a dilution of 1:2.5. No agglutinins for group O and sheep erythrocytes could be demonstrated in any of the 6 animals. After immunization with Type I pneumococcal vaccine the agglutinins for group AB increased in all animals and could be demonstrated up to dilutions as high as 1:20 in 3 sera. Agglutinins for group A showed only a slight increase. Antibodies against group O cells were found in a single blood sample of 2 rabbits. No sheep-blood agglutinins were present in the sera after immunization.

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[1] von Haam, E., and Rosenfeld, Irene, *J. Immunol.*, 1942, **43**, 109.

[2] Powell, G. H., and Jamieson, W. A., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 248.

[3] Levine, P., Bullowa, J. G. M., and Katzin, E. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 617.

During 2 weeks of hormone-administration the titer of agglutinins for group AB increased to 1:40 in the serum of 1 rabbit and to 1:20 in the sera of 4 others. The titer for group A rose to 1:40 in the serum of 1 rabbit and to 1:20 in the sera of 7 rabbits injected with estrone. Anti-O and antisheep agglutinins were demonstrated in the sera of all the rabbits that were tested. Following immunization with the vaccine an additional increase in the hemagglutinins was observed. The titers for AB cells were as high as 1:100, and those for group A cells as high as 1:80. Agglutinins for groups B and O and sheep erythrocytes were present in dilutions up to 1:20.

In Table I the average antibody-titers of animals which did and did not receive estrone before immunization with Type I pneumococcus have been compared. It appears quite evident that the process of immunization produced a marked increase in hemagglutinins in the rabbits which had received the female sex hormone as compared with the control animals. Moreover, it was shown that the administration of pure crystalline estrone alone increased the titer of nonspecific antibodies in the sera of the injected animals. Absorption-experiments are now under way to study the nature of these antibodies and to determine whether pure crystalline estrone resembles the Forssman antigen.

*Summary.* Administrations of estrone produced a moderate increase in the titer of nonspecific hemagglutinins in rabbits. This was followed by a more striking increase in the antibodies after immunization with pneumococcal vaccine.

TABLE I.
Average Titer of Heterophil Antibodies.

| Type of agglutinin | Experimental animals | | | Control animals | | |
|---|---|---|---|---|---|---|
| | Before exper. | After estrone injec. | After immun. | Before exper. | After sesame oil | After immun. |
| AB | 2.8 | 13.8 | 59.2 | 2.5 | 3.2 | 8.1 |
| A | 2.5 | 13.6 | 29.9 | 2.5 | 2.2 | 3.6 |
| B | 0 | 2.8 | 5.0 | 0.4 | 0.4 | 0.4 |
| O | 0 | 4.4 | 8.3 | 0 | 0 | 0.1 |
| Sheep | 0 | 6.9 | 11.7 | 0 | 0 | 0 |

## 13679 P

# Therapeutic Effect in Guinea Pigs of Hyperimmune Epidemic Typhus Antiserum.

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*From the Lederle Laboratories, Inc., Pearl River, N.Y.*

Although some studies[1] have been made of the properties of the sera of animals hyperimmunized with murine-typhus rickettsiæ, it has only recently been possible to get enough epidemic-typhus rickettsiæ for the preparation of a promising epidemic-typhus antiserum. A year ago we[2] pointed out that an antiserum of high neutralizing power could be produced by injecting rabbits with yolk-membrane suspensions from chick embryos infected with Breinl strain of typhus rickettsiæ inoculated according to the method of Cox.[3] A similar serum[4] made by injecting horses and donkeys with epidemic-typhus-diseased mouse-lungs has also been described. The present note records some preliminary studies of the therapeutic value in guinea pigs of our rabbit antiserum.

Topping[5] has shown that a good Rocky Mountain spotted-fever antiserum has a clearly demonstrable therapeutic value when injected into infected guinea pigs and monkeys. His original serum was made with an antigen prepared from infected ticks, but we have found[2] that a serum of powerful neutralizing titer can be made from spotted fever-infected egg-membranes. It is easy to show that rabbit antiserum prepared with this egg-propagated antigen has the same therapeutic value in guinea pigs that Topping[5] has described for the tick-antigen serum. Administration of these hyperimmune sera to guinea pigs infected with a virulent strain of Rocky Mountain spotted fever does not immediately suppress the febrile reaction and other clinical symptoms of the disease; instead, it shortens the febrile reaction and leads to prompt recovery of animals which otherwise would usually die. A similar modifying, rather than immediately curative, action is to be expected from the administration of typhus antiserum to epidemic typhus-diseased guinea pigs. Our experiments clearly demonstrate that such a positive therapeutic effect can be obtained.

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[1] Nicolle and Blaizot, *Ann. Inst. Pasteur*, 1916, **30**, 446; Zinsser, H., and Castaneda, *J. Exp. Med.*, 1933, **57**, 391.

[2] Kurotchkin, T. J., van der Scheer, J., and Wyckoff, R. W. G., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 323.

[3] Cox, H. R., *U. S. Public Health Rep.*, 1938, **53**, 2241.

[4] Durand, P., and Balozet, L., *Arch. Inst. Pasteur* (Tunis), 1940, **29**, 363; 1941, **30**, June.

[5] Topping, N. H., *U. S. Public Health Rep.*, 1940, **55**, 41.

In these experiments, guinea pigs have been infected by the intra-abdominal injection of typhus-diseased guinea-pig brain (Breinl strain*). Some of these infected animals were left untreated to serve as controls. Others received a single subcutaneous injection of ½ cc of a refined hyperimmune rabbit-serum concentrate (total protein = 7%). This serum was administered after an interval of

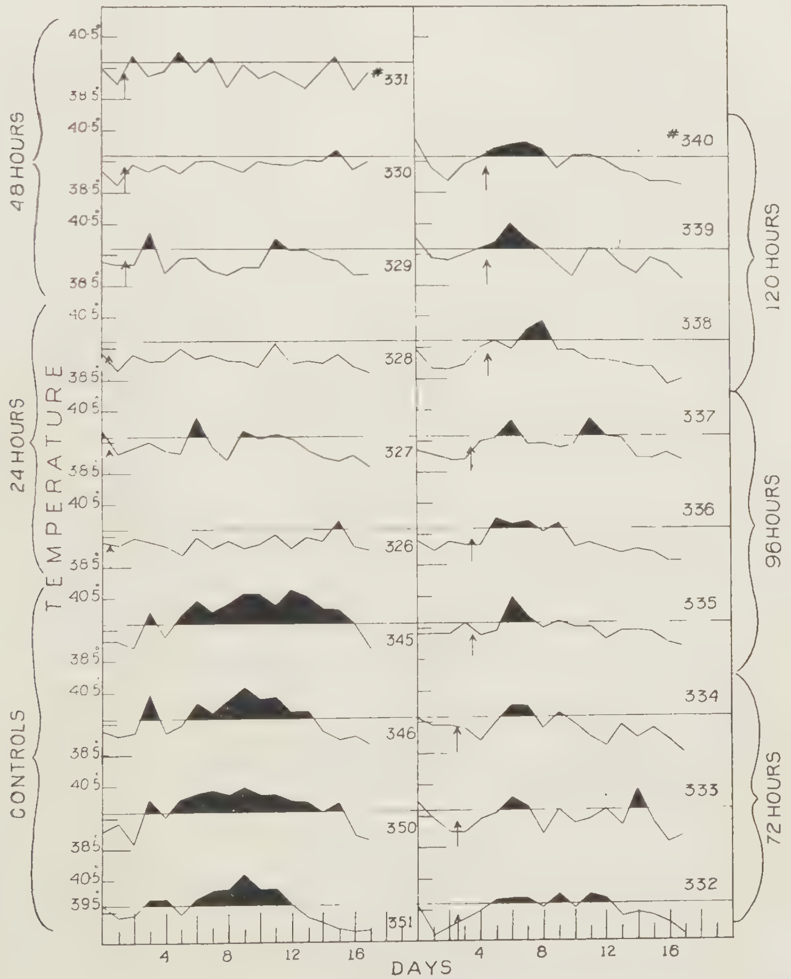

Fig. 1.

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* Obtained from the U. S. National Institute of Health and carried serially through guinea pigs.

TABLE I.

| Guinea pig No. | cc of serum | Serum × hrs after infection, hrs | Area under fever curve | Guinea pig No. | cc of serum | Serum × hrs after infection, hrs | Area under fever curve |
|---|---|---|---|---|---|---|---|
| U 45 | 0 | — | 173 | 344 | 0 | — | 144 |
| 46 | 0 | — | 152 | 345 | 0 | — | 254 |
| 47 | 0 | — | 165 | 346 | 0 | — | 133 |
| 48 | 0 | — | 88 | 347 | 0 | — | 101 |
| 49 | 0 | — | 173 | 349 | 0 | — | 178 |
| 50 | 0 | — | 110 | 350 | 0 | — | 150 |
| 51 | 0 | — | 165 | 351 | 0 | — | 121 |
| 52 | 0 | — | 252 | 326 | .5 | 24 | 0 |
| 53 | .5 | 24 | 30 | 327 | .5 | 24 | 4 |
| 54 | .5 | 24 | 20 | 328 | .5 | 24 | 0 |
| 55 | .5 | 24 | 5 | 329 | .5 | 48 | 22 |
| 56 | .5 | 24 | 12 | 330 | .5 | 48 | 0 |
| 57 | .5 | 48 | 2 | 331 | .5 | 48 | 16 |
| 58 | .5 | 48 | 10 | 332 | .5 | 72 | 32 |
| 59 | .5 | 48 | 0 | 333 | .5 | 72 | 30 |
| 60 | .5 | 48 | 26 | 334 | .5 | 72 | 18 |
| 61 | .5 | 72 | 31 | 335 | .5 | 96 | 26 |
| 62 | .5 | 72 | 40 | 336 | .5 | 96 | 18 |
| 63 | .5 | 72 | 12 | 337 | .5 | 96 | 21 |
| 64 | .5 | 72 | 30 | 338 | .5 | 120 | 28 |
| 65 | .5 | 96 | 19 | 339 | .5 | 120 | 39 |
| 66 | .5 | 96 | 20 | 340 | .5 | 120 | 36 |
| 67 | .5 | 96 | 0 | | | | |
| 68 | .5 | 96 | 0 | Avg | 0 | — | 155 |
| | | | | ,, | .5 | 24 | 1 |
| Avg | 0 | — | 160 | ,, | .5 | 48 | 13 |
| ,, | .5 | 24 | 17 | ,, | .5 | 72 | 27 |
| ,, | .5 | 48 | 10 | ,, | .5 | 96 | 22 |
| ,, | .5 | 72 | 28 | ,, | .5 | 120 | 34 |
| ,, | .5 | 96 | 10 | | | | |

from one to 5 days following injection of virus. Temperatures were taken on all animals for a period of 18 days and the height and duration of the febrile reaction were taken as a measure of the severity of infection. In all experiments a uniform infecting dose of virus was used. This consisted of 1 cc of a freshly prepared 10% suspension in saline of a guinea-pig brain harvested on the third day of fever. In the present series of experiments serum was administered from 1 to 5 days after infection. Other experiments are now under way in which serum is being given at still longer intervals following virus administration.

Three experiments have thus far been carried out, with similar results in each case. Febrile curves from one series are reproduced in Fig. 1. It is obvious from them that serum administered after infection has a well-defined effect upon the course of an epidemic-typhus infection in the guinea pig. In order to find out how great an interval may be allowed to elapse between infection and injection of serum before this effect is no longer demonstrable, it is clearly necessary to have some sort of quantitative measure of the febrile reaction. This is most simply obtained by determining with a planimeter areas under the febrile portion of the temperature-curve. Such areas from the experimental series illustrated in Fig. 1 and from a second similar series are listed in Table I. Throughout this work a guinea pig is considered to be febrile whenever its temperature exceeds 39.7°C. The data from the third series of experiments are less complete because of seriously interfering intercurrent infection in the available guinea pigs; none of the results of this third series, however, conflicts in any respect with those outlined above. It is obvious from the foregoing that serum administered as much as 5 days following infection has a positive therapeutic effect in guinea pigs diseased with epidemic typhus.

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## 13680 P

## Lesions Produced with Sulfadiazine.*

BERNARD MAISEL, BARTON MCSWAIN AND FRANK GLENN.
(Introduced by W. Dock.)

*From the Departments of Pathology and Surgery, New York Hospital, and the Cornell University Medical College.*

This study was undertaken to note the effects of sulfadiazine on the tissues of the dog.

Changes apparently due to the sulfonamides have been reported in man and in experimental animals by several investigators.[1-7] Some of the changes observed by us have not been hitherto described.

Eight dogs, weighing from 8.2 to 16.4 kg, were used. In a 20% aqueous solution, one-tenth g of sodium sulfadiazine per kg of animal weight was injected subcutaneously twice daily. Tissues were examined from 6 to 21 days after the initial dose of this drug.

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* The sodium sulfadiazine used in this experiment was supplied by Lederle Laboratories, Inc.

[1] French, A. J., and Weller, C. V., *Am. J. Path.*, 1942, **1**, 109.

[2] Antopol, W., and Robinson, H., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 428; *Ibid.*, *Arch. Path.*, 1940, **29**, 67.

[3] Antopol, W., Lehr, D., Churg, J., and Sprinz, H., *Arch. Path.*, 1941, **31**, 592.

[4] Stryker, W. A., *J. A. M. A.*, 1940, **114**, 953.

[5] Davis, H. A., and Higgins, G. M., *Am. J. M. Sc.*, 1939, **198**, 804.

[6] Toomey, J. A., Reichle, H. S., and Takacs, W. S., *J. Pediat.*, 1940, **16**, 179.

[7] Machella, T. E., and Higgins, G. M., *Am. J. M. Sc.*, 1939, **198**, 804.

During the first 6 days blood levels were from 10 to 25 mg %; higher levels (up to 100 mg %) were found after the second week. All animals developed high values for blood urea nitrogen, became listless, and 6 died within 21 days. The 2 which remained were killed.

A moderate bronchopneumonia was found in 2 animals. There were no other gross changes except for the presence of crystalline deposits in the pelves and calyces of all animals.

The most striking changes in all experimental animals were found in the kidneys. This drug apparently affects several renal structures at approximately the same time. The lesions indicated below were present after 6 days, and with increased time the characteristics became more obvious.

A moderate number of glomeruli were damaged. The first evidence of damage was the presence of protein and blood elements in Bowman's space and the urine. Later, inflammatory changes were encountered resulting in the destruction of the glomeruli. The leukocytic reaction consisted of monocytes, plasma cells, lymphocytes and a few polymorphonuclear neutrophils and eosinophils. This reaction was accompanied by the development of fibroblasts, collagen deposition and multinucleated giant cells producing a granuloma.

The tubular system was simultaneously affected. Metachromatic, often quite basophilic, protein-sulfadiazine crystalline casts were found as high as the descending loop of Henle. Many of the tubules, particularly the proximal and distal convoluted tubules, showed swelling and early necrosis of the lining cells, with a mononuclear leukocytic reaction in the regional stroma. In many areas materials in tubular casts had broken through the necrotic part of the tubular wall so as to permeate the interstitial tissues. About this material a leukocytic reaction similar to that described in the glomerular lesion developed. After approximately 3 weeks multinucleated giant cells, epithelioid cells, fibroblasts and collagen appeared, forming a granuloma.

Casts of proteins and crystals often completely filled and obstructed many collecting tubules in medullary pyramids. Some lining cells showed evidence of early necrosis, and regenerative response as disclosed by mitotic figures was a conspicuous feature.

In the submucosa of the pelvis and calyces there was an inflammatory reaction consisting of the same cells that occurred in the inflammatory lesions in the cortex and medulla.

An inflammatory reaction occurred in and about the walls of several veins of the kidney. The leukocytes were largely mononuclear in type, with occasional polymorphonuclear eosinophils and neutrophils. Where the inflammatory reaction was found in the vessel wall a mural thrombus was often attached. Many small veins were completely thrombosed, and hemorrhage had occurred into the interstitial tissues of the kidney.

About several of the arteries of the kidney and occasionally of the heart and liver there was a perivascular leukocytic reaction of monocytes, lymphocytes, plasma cells and occasionally a few polymorphonuclear eosinophils and neutrophils.

The sternal bone marrow of 4 animals showed a moderate hyperplasia of all elements. The other 4 animals showed either no change or a slight hypoplasia. One animal had small areas of necrosis in the bone marrow. Two animals showed small areas of hemorrhage in the sternal marrow. In the marrow of all animals there were many mononuclear phagocytic cells filled with hemosiderin.

In the spleen there was a large amount of intracellular and extracellular hemosiderin. All animals showed a prominent "toxic" change of the germinal centers.

In 7 animals the Kupffer cells of the liver were filled with hemosiderin.

*Discussion.* These findings indicate that sodium sulfadiazine produces several types of lesions in dogs. Those occurring about tubules, calyces and renal pelves are probably due to the chemical irritation in these parts. Damage to the glomeruli and tubules often terminates in the destruction of these structures with occasional production of a granulomatous lesion characterized by mononuclear leukocytes, multinucleated giant cells, fibroblasts and collagen deposition. The hemosiderosis in the bone marrow, spleen and liver is probably secondary to the destruction of erythrocytes. The hyperplasia of the bone marrow in 4 dogs is a response to the destruction of erythrocytes. The hemorrhage, repression of activity and necrosis of bone marrow in some animals is unexplained, as are the vascular lesions, including those of the glomeruli.

### 13681

## Agglutinogens in Fetal Erythrocytes.

S. Bornstein and M. Israel. (Introduced by Ella H. Fishberg.)

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While statements in the older literature made it appear questionable whether the individual blood group is always developed in the newborn, there is now no doubt that with proper technic the agglutinogens A and B in the red cells can always be detected. That the same applies to the factors M and N is evident from the fact that in many thousands of tests performed for legal purposes no individual has been found whose cells contained neither M nor N, nor a case where the blood types of mother and newborn were incompatible with the laws of heredity as established by Landsteiner and Levine.

Little, however, is known concerning how early in fetal life all the agglutinogens can be detected by ordinary agglutination tests. Moureau[1] demonstrated the factors M and N in very young fetuses. Hyman[2] found M and N demonstrable in 3 fetuses but failed to obtain either M or N agglutination with the cells of a fourth, 3 months old, whose cells exhibited the agglutinogen B. She left the question open whether agglutinogens had disintegrated before expulsion of the fetus or whether they had not yet developed at that time. Recently, she reported on 4 premature newborns, 2 born at 7 and 2 at 8 months.[3]

It seemed worth while examining a somewhat larger series of fetuses to determine whether or not their agglutinogens are detectable with sera and testing fluids as they are used in transfusion work and in legal tests. The result would give further weight to the acknowledged fact that determinations of blood group and blood type in young infants are dependable. Doubt is occasionally still being cast upon this fact by lay opponents to the legal applications of blood tests, which unfortunately still do not enjoy quite general recognition.[4] In addition, by including the Rh factor in the investigation, information could be obtained on the value of tests performed with fetal blood for investigations on isoimmunization as a cause of habitual abortion according to Levine.[5]

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[1] Moureau, P., *Rev. belg. des Scienc. Méd.*, 1935, **7**, 540.

[2] Hyman, H. S., *Abstr. Doct. Diss.*, No. 21, The Ohio State University Press, 1937.

[3] Hyman-Parker, H. S., *J. Immun.*, 1942, **43**, 1.

[4] Schatkin, S. B., *J. Crim. Law and Criminol.*, 1941, **32**, 458.

*Materials and Technic.* A series of 19 premature fetuses was examined in the order in which they came to the pathological examination, only those showing gross evidence of maceration being omitted. The heart was tapped and a suspension of the blood in physiologic saline solution (about 1% cells) was prepared and examined the same day with the centrifuge method. In some cases examination of parents was possible. Various human A and B sera and M and N testing fluids were used, which gave positive agglutinations with the corresponding cells in dilution 1:8. A small amount of human anti-Rh serum was available through the courtesy of Dr. Philip Levine. For the Rh tests the test tubes were incubated for one hour at 37°C before centrifugation.

*Results.* As may be seen from Table I, the fetuses varied in length between 7 and 50 cm, the average being 28.6 cm. The distribution of agglutinogens was independent of the length of the fetuses, and did not grossly deviate from the percentages in the population. No fetus without either M or N was encountered, and no combination parent M, fetus N or *vice versa* occurred. The Rh factor was demonstrable in 4 out of 5 fetuses. This confirms the

TABLE I.
Blood Group and Blood Type of Fetuses and Parents. Presence or Absence of Rh Factor (Tested in No. 15-19 Only).

| Fetus | | | | |
|---|---|---|---|---|
| No. | Length | Agglutinogens | Mother | Father |
| 1 | 21 cm | O MN | O M | |
| 2 | 7 cm | O MN | B M | |
| 3 | 13 cm | A M | A M | |
| 4 | 47 cm | O MN | | |
| 5 | 23 cm | A M | | |
| 6 | 13 cm | O M | O MN | O MN |
| 7 | 30 cm | A MN | A MN | A MN |
| 8 | 50 cm | A M | | |
| 9 | 42 cm | A N | | |
| 10 | 27 cm | A MN | | |
| 11 | 19 cm | O N | | |
| 12 | 26 cm | B M | | |
| 13 | 30 cm | A N | | |
| 14 | 33 cm | B N | A MN | |
| 15 | 41 cm | AB M Rh+ | A MN Rh+ | |
| 16 | 42 cm | A M Rh+ | O MN Rh+ | |
| 17 | 39 cm | A MN Rh— | A MN Rh— | |
| 18 | 17 cm | O N Rh+ | A N Rh+ | |
| 19 | 21 cm | B MN Rh+ | A MN Rh— | B N Rh+ |

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[5] Levine, P., Burnham, L., Katzin, E. M., and Vogel, P., *Am. J. Obst. and Gyn.*, 1941, **42**, 925.

indirect evidence that the Rh factor is present early in fetal life as derived from Levine's work.

*Summary and Conclusions.* Agglutination tests with the heart's blood of 19 fetuses show that with ordinary technic, *i. e.*, using sera and testing fluids with agglutinin titers of at least 1:8 and not too strong cell suspensions, agglutinations for A, B, M, N, and Rh can be obtained. This indicates that the agglutinogens are developed long before birth in sufficient strength for reliable examination.

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